Universidade de Lisboa

Faculdade de Farmácia

MICROALGAE AS BIOINDICATORS FOR WATER QUALITY ANALYSES

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Mestrado Integrado em Ciências Farmacêuticas

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Resumo

Milhares de novos poluentes e seus metabolitos encontram-se omnipresentes em todos os compartimentos aquáticos à escala mundial sem que se conheça bem o seu comportamento e toxicidade, nomeadamente riscos para o ambiente e saúde pública.

Benzenosulfonamidas, benzotiazóis e benzotriazóis são compostos com um grande volume de produção que devido às suas várias aplicações a nível industrial e utilização em vários produtos de uso habitual, combinado com a sua elevada solubilidade em água e resistência à biodegradação são já considerados como novos poluentes aquáticos a nível global. Estes contaminantes foram encontrados em vários compartimentos aquáticos em concentrações desde alguns ng/L até centenas de µg/L. É por isso imprescindível encontrar-se o melhor método de detecção destes poluentes, nomeadamente ao nível de rapidez de análise e custo-efectividade.

Estes poluentes são habitualmente analisados por métodos químicos que apesar da sua elevada sensibilidade apresentam várias desvantagens nomeadamente o seu custo e tempo. A utilização de biosensores para monitorização ambiental é ideal pelo facto de serem métodos rápidos, custo-efectivos, simples e podem ser utilizados *"on-site"* para detecção e análise em tempo real dos contaminantes no local. Neste estudo foram utilizadas microalgas como biosensores por terem tempos de resposta rápidos, reprodutibilidade, requererem pouco ou nenhum tratamento das amostras em análise (pelo que podem ser utilizados directamente no local para detecção dos contaminantes), apresentarem elevada sensibilidade e serem bons marcadores biológicos.

Assim, o objectivo deste trabalho foi avaliar a sensibilidade de três microalgas diferentes a sete novos poluentes (BSA, pTSA, BT, MeSBT, HOBT, BTR e 5TTR) por testes de inibição de crescimento (que foram executados utilizando espectrofotometria e observação macroscópica) com o objectivo de utilizar estas microalgas no futuro como biosensores para detecção e monitorização destes sete contaminantes em diferentes compartimentos aquáticos.

Os resultados neste estudo mostraram que o crescimento da microalga Ph não é afectado pela presença das benzenosulfonamidas em estudo no meio. Os efeitos do BSA no crescimento da microalga K, do BT no das microalgas D e K e do HOBT no

da microalga Ph foram inconclusivos, principalmente pela falta de concordância dos dois testes realizados para cada par microalga-poluente, pelo que os seus efeitos devem ser estudados em futuros estudos.

A microalga D foi considerada a melhor escolha como futuro biossensor para a execução de futuros controlos de qualidade das águas nos quais o BSA se suspeita estar presente em concentrações iguais ou superiores a 25ppm. Os resultados para o pTSA mostraram que neste caso a melhor escolha como biossensor seria igualmente a microalga D que apresentou uma sensibilidade de 1ppm para este poluente. A microalga Ph foi considerada a melhor escolha como biossensor para ensaios de monitorização e detecção dos poluentes BT, MeSBT, BTR e 5TTR na água, para os quais o crescimento da microalga foi afectado a partir de concentrações de 10ppm, 25ppm, 25ppm e 5ppm respectivamente. A microalga K apresentou a maior sensibilidade para o HOBT (de 10ppm) pelo que esta seria a melhor escolha como futuro biossensor para este contaminante.

De acordo com a classificação da Comissão das Comunidades Europeias, utilizando os valores de EC_{50} estimados neste estudo, o BSA foi considerado prejudicial para a microalga D tal como o pTSA para as microalgas D e K. O BT foi também classificado como prejudicial para a Ph, o poluente MeSBT como não tóxico para a microalga D e como prejudicial para as microalgas K e Ph e o HOBT também como prejudicial para ambas microalgas D e K. O BTR foi considerado prejudicial para as três microalgas e o poluente 5TTR como prejudicial para ambas K e Ph microalgas e como não tóxico para a D.

Palavras-chave: Microalgas; Biosensores; Benzenosulfonamidas; Benzotiazois; Benzotriazois.

Abstract

Benzenesulfonamides, benzothiazoles and benzotriazoles are high-volume production chemicals that are already considered to be ubiquitous water contaminants. These emerging pollutants are commonly analysed by chemical methods however, biosensors have the advantages of being highly sensitive, simple, fast, cheap and can be used for on-site analysis.

We used three different microalgae as biosensors and evaluated their sensitivity to the BSA, BT and BTR and some of their derivatives by performing algal growth inhibition assays in order to use them in the future for detection and monitoring water environments.

The results showed the Ph microalga isn't sensitive to the benzenesulfonamides in study. The effects of the BSA on the growth of the K microalga, the BT on both D and K microalgae and HOBT on Ph microalga were inconclusive. The D microalga was considered the best choice as biosensor to analyses involving both BSA and pTSA with sensitivities of 25ppm and 1ppm, respectively. The Ph microalga would be the best choice as a biosensor to analyse the presence of both BT and MeSBT in the water, due to its sensitivities of 10ppm and 25ppm respectively, and the K microalga for the HOBT with a sensitivity of 10ppm. The Ph microalga was the most sensitive to both BTR and 5TTR with sensitivities of 25ppm and 5ppm respectively.

With the EC_{50} values estimated in this study, the BSA was considered harmful to the D microalga as well as the pTSA for the D and K microalgae. The BT was also classified as harmful for the Ph, the pollutants MeSBT and 5TTR as non-toxic for the D microalga and as harmful for the K and Ph and the HOBT also as harmful for both D and K microalgae. The BTR was considered harmful for all three microalgae.

Keywords: Microalgae; Biosensors; Benzenesulfonamides; Benzothiazoles; Benzotriazoles.

Acronyms

- Microalga K – *Pseudokirchneriella subcapitata*
- Microalga Ph – *Phaeodactylum tricornutum*
- Microalga D – *Dunaliella tertiolecta*
- BSA – benzenesulfonamide
- pTSA – *para*-toluenesulfonamide
- BT – benzothiazole
- MeSBT – 2-(methylthio)benzothiazole
- HOBT – 2-hydroxybenzothiazole
- BTR – benzotriazole
- 4TTR – 4-methylbenzotriazole
- 5TTR – 5-methylbenzotriazole
- ppm – parts per million
- ppb – parts per billion
- ROS – reactive oxygen species
- EC_{50} – effective concentration

Table of contents

1 Introduction 11
1.1 Benzenesulfonamides 13
1.2 Benzotriazoles 14
1.3 Benzothiazoles 15
1.4 Microalgae as biosensors 16
1.4.1 *Dunaliella tertiolecta* 18
1.4.2 *Phaeodactylum tricornutum* 18
1.4.3 *Pseudokirchneriella subcapitata* 19
1.5 Spectrophotometry 19
2 Materials and methods 21
2.1 Equipment 21
2.2 Materials 21
2.3 Microalgae 21
2.4 Reagents 21
2.5 Experimental procedures 23
2.5.1 Microalgae cultures 23
2.5.2 Samples and controls solutions 24
2.5.3 Growth inhibition assays 24
2.5.4 Cell counting 25
2.5.5 Data analysis 25
3 Results 26
3.1 Benzenesulfonamides toxicity 27
3.1.1 Benzenesulfonamide 27
3.1.1.1 *Dunaliella tertiolecta* 27
3.1.1.2 *Pseudokirchneriella subcapitata* 29
3.1.1.3 *Phaeodactylum tricornutum* 31
3.1.2 *para*-toluenesulfonamide 32
3.1.2.1 *Dunaliella tertiolecta* 32
3.1.2.2 *Pseudokirchneriella subcapitata* 34
3.1.2.3 *Phaeodactylum tricornutum* 36
3.2 Benzothiazoles toxicity 38
3.2.1 Benzothiazole 38
3.2.1.1 *Dunaliella tertiolecta* 38
3.2.1.2 *Pseudokirchneriella subcapitata* 40
3.2.1.3 *Phaeodactylum tricornutum* 42
3.2.2 2-hydroxybenzothiazole 44
3.2.2.1 *Dunaliella tertiolecta* 44
3.2.2.2 *Pseudokirchneriella subcapitata* 45
3.2.2.3 *Phaeodactylum tricornutum* 47
3.2.3 2-(methylthio)benzothiazole 50
3.2.3.1 *Dunaliella tertiolecta* 50
3.2.3.2 *Pseudokirchneriella subcapitata* 51
3.2.3.3 *Phaeodactylum tricornutum* 54
3.3 Benzotriazoles toxicity 56
3.3.1 Benzotriazole 56
3.3.1.1 *Dunaliella tertiolecta* 56
3.3.1.2 *Pseudokirchneriella subcapitata* 57
3.3.1.3 *Phaeodactylum tricornutum* 60

3.3.2 5-methylbenzotriazole 61
3.3.2.1 *Dunaliella tertiolecta* 61
3.3.2.2 *Pseudokirchneriella subcapitata* 63
3.3.2.3 *Phaeodactylum tricornutum* 66
4 Discussion 68
5 Conclusions 71
References 73

Captions

Figure 1 – Molecular structure of the benzenesulfonamides studied in this work 13
Figure 2 - Molecular structure of the benzotriazoles studied in this work 15
Figure 3 - Molecular structure of the benzothiazoles studied in this work 16
Figure 4 – Microscopic visualisation of the three microalgae used in this study 19
Figure 5 – Illustration of a double-beam spectrophotometer (44) 20
Figure 6 – Microalgae culture chamber in the laboratory. 23
Figure 7 – First test of microalga D growth rates with different concentrations of BSA in the medium 27
Figure 8 – Second test of microalga D growth rates with different concentrations of BSA in the medium 28
Figure 9 – Samples and control tubes of the first test (left) and second test (right) of the D microalga culture with BSA on the last day of the test 28
Figure 10 – First test of microalga K growth rates with different concentrations of BSA in the medium 29
Figure 11 – Second test of microalga K growth rates with different concentrations of BSA in the medium 30
Figure 12 – Samples and control tubes of the first test (left) and second test (right) of the K microalga culture with BSA on the last day of the test 30
Figure 13 – First test of microalga Ph growth rates with different concentrations of BSA in the medium 31
Figure 14 – Second test of microalga Ph growth rates with different concentrations of BSA in the medium 32
Figure 15 – Samples and control tubes of the first test (left) and second test (right) of the Ph microalga culture with BSA on the last day of the test 32
Figure 16 – First test of microalga D growth rates with different concentrations of pTSA in the medium 33
Figure 17 – Second test of microalga D growth rates with different concentrations of pTSA in the medium 33
Figure 18 – Samples and control tubes of the first test (left) and second test (right) of the D microalga culture with pTSA on the 32nd and 22nd days, respectively 34
Figure 19 – First test of microalga K growth rates with different concentrations of pTSA in the medium 35
Figure 20 – Second test of microalga K growth rates with different concentrations of pTSA in the medium 35
Figure 21 – Samples and control tubes of the first test (left) and second test (right) of the K microalga culture with pTSA on the last day of the test and on the 22nd day, respetively 36
Figure 22 – First test of microalga Ph growth rates with different concentrations of pTSA in the medium 37

Figure 23 – Second test of microalga Ph growth rates with different concentrations of pTSA in the medium 37
Figure 24 – Samples and control tubes of the first test (left) and second test (right) of the Ph microalga culture with pTSA on the last day of the test 38
Figure 25 – First test of microalga D growth rates with different concentrations of BT in the medium 38
Figure 26 – Second test of microalga D growth rates with different concentrations of BT in the medium 39
Figure 27 – Samples and control tubes of the first test (left) and second test (right) of the D microalga culture with BT on the 34th and 25th days, respectively 40
Figure 28 – First test of microalga K growth rates with different concentrations of BT in the medium 41
Figure 29 – Second test of microalga K growth rates with different concentrations of BT in the medium 41
Figure 30 – Samples and control tubes of the first test (left) and second test (right) of the K microalga culture with BT on the last day of the test 42
Figure 31 – First test of microalga Ph growth rates with different concentrations of BT in the medium 42
Figure 32 – Second test of microalga Ph growth rates with different concentrations of BT in the medium 43
Figure 33 – Samples and control tubes of the first test (left) and second test (right) of the Ph microalga culture with BT on the last day of the test 43
Figure 34 – First test of microalga D growth rates with different concentrations of HOBT in the medium 44
Figure 35 – Second test of microalga D growth rates with different concentrations of HOBT in the medium 45
Figure 36 – Samples and control tubes of the first test (left) and second test (right) of the D microalga culture with HOBT on the last day of the test 45
Figure 37 – First test of microalga K growth rates with different concentrations of HOBT in the medium 46
Figure 38 – Second test of microalga K growth rates with different concentrations of HOBT in the medium 46
Figure 39 – Samples and control tubes of the first test (left) and second test (right) of the K microalga culture with HOBT on the 21st and 22nd days, respectively 47
Figure 40 – First test of microalga Ph growth rates with different concentrations of HOBT in the medium 48
Figure 41 – Second test of microalga Ph growth rates with different concentrations of HOBT in the medium 49
Figure 42 – Samples and control tubes of the first test (left) and second test (right) of the Ph microalga culture with HOBT on the 19th and 22nd days, respectively 49
Figure 43 – First test of microalga D growth rates with different concentrations of MeSBT in the medium 50
Figure 44 – Second test of microalga D growth rates with different concentrations of MeSBT in the medium 51
Figure 45 – Samples and control tubes of the first test (left) and second test (right) of the D microalga culture with MeSBT on the last day of the test 51
Figure 46 – First test of microalga K growth rates with different concentrations of MeSBT in the medium 52
Figure 47 – Second test of microalga K growth rates with different concentrations of MeSBT in the medium 53

Figure 48 – Samples and control tubes of the first test (left) and second test (right) of the K microalga culture with MeSBT on the last day of the test 53
Figure 49 – First test of microalga Ph growth rates with different concentrations of MeSBT in the medium 54
Figure 50 – Second test of microalga Ph growth rates with different concentrations of MeSBT in the medium 55
Figure 51 – Samples and control tubes of the first test (left) and second test (right) of the Ph microalga culture with MeSBT on the 21st and 29th days, respectively ... 55
Figure 52 – First test of microalga D growth rates with different concentrations of BTR in the medium 56
Figure 53– Second test of microalga D growth rates with different concentrations of BTR in the medium 57
Figure 54 – Samples and control tubes of the first test (left) and second test (right) of the D microalga culture with BTR on the 34th and 25th days, respectively 57
Figure 55 – First test of microalga K growth rates with different concentrations of BTR in the medium 58
Figure 56 – Second test of microalga K growth rates with different concentrations of BTR in the medium 59
Figure 57 – Samples and control tubes of the first test (left) and second test (right) of the K microalga culture with BTR on the last day of the test 59
Figure 58 – First test of microalga Ph growth rates with different concentrations of BTR in the medium 60
Figure 59 – Second test of microalga Ph growth rates with different concentrations of BTR in the medium 61
Figure 60 – Samples and control tubes of the first test (left) and second test (right) of the Ph microalga culture with BTR on the last day of the test 61
Figure 61 – First test of microalga D growth rates with different concentrations of 5TTR in the medium 62
Figure 62 – Second test of microalga D growth rates with different concentrations of 5TTR in the medium 63
Figure 63 – Samples and control tubes of the first test (left) and second test (right) of the D microalga culture with 5TTR on the last day of the test 63
Figure 64 – First test of microalga K growth rates with different concentrations of 5TTR in the medium 64
Figure 65 – Second test of microalga K growth rates with different concentrations of 5TTR in the medium 65
Figure 66 – Samples and control tubes of the first test (left) and second test (right) of the K microalga culture with 5TTR on the 14th and 15th days, respectively 65
Figure 67 – First test of microalga Ph growth rates with different concentrations of 5TTR in the medium 66
Figure 68 – Second test of microalga Ph growth rates with different concentrations of 5TTR in the medium 67
Figure 69 – Samples and control tubes of the first test (left) and second test (right) of the Ph microalga culture with 5TTR on the 19th and 22nd days, respectively 67

1 Introduction

Water is one of the most important natural resources however, mostly because of the human activities, its natural balance is being increasingly disrupted. Water pollution is a main concern worldwide not only because of human health issues but also the negative impact on animals, marine environment and freshwater organisms.

The pressure by citizens and environmental organizations for cleaner rivers, lakes, groundwater and coastal waters led to the "European Water Framework Directive" which was adopted in 2000. This new water framework directive focused mainly on setting water quality standards, implementing new discharge controls and minimizing the impacts of anthropogenic pressures on surface water quality (1).

A water pollutant can be defined as a physical, chemical or biological factor causing aesthetic or detrimental effects on aquatic life and on those who consume water. Most of the water pollutants are chemicals which remain suspended or dissolved in water (2). Some of these water pollutants have been extensively investigated through the years, so the adverse effects on the environment and human health are well-known, however, over a thousand emerging pollutants and their metabolites are found ubiquitously in all aquatic compartments exceeding sometimes the safety threshold for predicted environmental concentrations in surface waters of 0.01 µg/L and their behaviour and ecotoxicological effects remain little known (3). It is necessary to create measures not only concerning the disposal and elimination of all water pollutants but also to improve the methods of analysis (4).

This continuous increasing amount of new potentially harmful pollutants in freshwater and marine environments calls for fast and cost-effective methods of analyses. Although chemical techniques are usually first choice for analysis of environmental contaminants because of their high sensitivity and selectivity, they have the main disadvantages of being time-consuming and expensive. Using

biosensors is ideal for environmental monitoring because they are cost-effective, rapid, easy and can be used on-site for real time detection and analysis of contaminants in the field (5,6).

Toxicity assays using microalgae as bioindicators have gained importance due to their simplicity, cost-effectiveness and reproducibility. These organisms play an important role in the sustainability of ecosystems: they provide food for higher trophic levels, produce oxygen and strongly influence the carbon cycle. Then, logically, if the phytoplankton is negatively affected by water pollutants, all of the ecosystem may also be implicated, either directly or indirectly because of the lack of food source. Due to all of these reasons, combined with the fact that microalgae are easy to culture and have ubiquitous distribution all over the world, makes them ideal test species for environmental toxicological studies (7–9).

Three different contaminants and their derivatives, which have already been classified as emerging pollutants, will be tested: benzenesulfonamides,, benzothiazoles and benzotriazoles.

These pollutants are high-volume production chemicals that due to their widespread use in everyday consumer products and many industrial applications along with their high water solubility and resistance to biodegradation makes them already considered to be ubiquitous water contaminants (10,11).

The benzothiazoles compounds, for example, have been detected in various environmental matrices including house dust, exhaled breath, adipose tissue and in 100% of human urine in several countries. Both benzothiazoles and benzotriazoles compounds were detected in a range of water types like surface water, drinking water and primary and secondary wastewaters. Benzenesulfonamide compounds were found in both river and sewage plants in concentrations up to µg/L in some cases. Several benzotriazoles, benzothiazoles and benzenesulfonamides (that included all the pollutants tested in this study) were found in environmental waters, such as surface water and sewage, at concentrations from a few ng/L to hundreds of µg/L. However, their toxicity and possible human health adverse effects as well as environmental dangers are not well understood yet (10–13).

The main goal of the present work is to assess the sensitivity of three different microalgae (*Dunaliella tertiolecta,, Phaeodactylum tricornutum* and

Pseudokirchneriella subcapitata) to three different contaminants (benzenesulfonamide, benzothiazole, benzotriazole) and some of their derivatives (*para*-toluenesulfonamide, 2-hydroxybenzothiazole, 2-(methylthio)benzothiazole, 5-methylbenzotriazole) by evaluating algal growth inhibition effects in order to use them as future biosensors for these emerging pollutants detection and monitoring in water environments.

1.1 Benzenesulfonamides

This class of chemical substances is the less studied of all the three classes that are being analysed in this study although they have widespread use in industry and households everyday just like the other two classes. Benzenesulfonamides have a benzene or toluene ring with a sulphonamide group substituent in which the formula of the parent compound is $C_6H_7O_2NS$ (11).

Benzenosulfonamides are usually used in the synthesis of pharmaceutical products, artificial fibres, colouring and plastic additives and as intermediate synthesis products for pesticides and saccharine. In this work we will evaluate the effect of two benzenesulfonamides on algal growth: the parent compound benzenesulfonamide (BSA) and one of its derivatives, the *para*-toluenesulfonamide (pTSA) (Figure 1). The parent compound BSA is mostly used in the synthesis of disinfectants, photochemical products and dyes, and pTSA as a plasticizer and as a fungicide in paints and coatings. There is not much information about benzenesulfonamides toxicity except for pTSA that was considered moderately toxic and the Organisation for Economic Co-operation and Development (OECD) has recommended additional tests if large amounts of pTSA will still be used in the future (10,11,14).

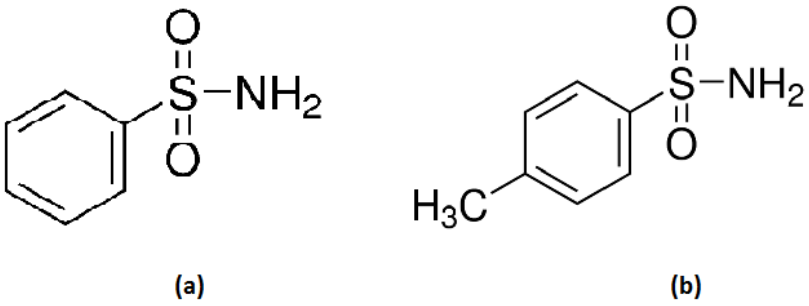


Figure 1 – Molecular structure of the benzenesulfonamides studied in this work

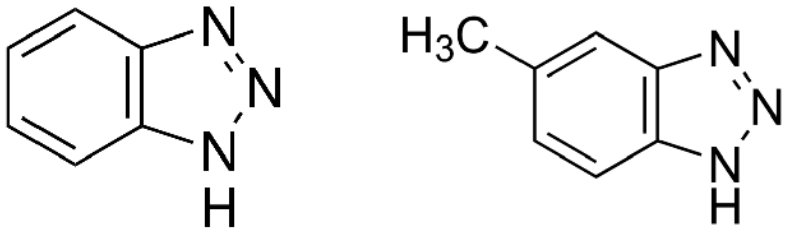
(a) benzenesulfonamide, (b) *para*-toluenesulfonamide

1.2 Benzotriazoles

Benzotriazoles are heterocyclic compounds in which a 1,2,3-triazole ring is condensed to a benzene ring and the chemical formula of the parent compound is $C_6H_5N_3$. The most common benzotriazoles are the parent compound benzotriazole (BTR) and the isomers 4- and 5-methylbenzotriazole (4TTR and 5TTR) (11,15). In this study we will only test the parent compound BTR and one of the isomers, the 5TTR (Figure 2).

Benzotriazoles can form a stable coordination compound with some metals and steels, especially copper and brass, conferring them anticorrosion properties, and so being widely used in metal finishing industry and in semiconductor industry and added to various fluids that come in contact with metals like de-icing and anti-icing fluids, cooling liquids, dishwasher detergents and brake fluids. They are also used as vulcanization accelerators in rubber production, as antifogging agents in photography and as intermediates for pharmaceuticals, dyes and fungicides. Some benzotriazoles derivatives have UV light stabilizing capacities so they can also be found in daily care products and in textiles and plastic materials, for example, including food and drinks containers, to preserve their integrity. Due to their wide spread use in industry and daily life they have an estimated worldwide production of 9000 tons/year (11,15–19).

Toxicological studies have demonstrated that benzotriazoles might be harmful to plants, mutagenic in bacteria cell systems and toxic to some microorganisms. BTR was classified by the Dutch Expert Committee on Occupational Standards as a suspected human carcinogen and the potential estrogenic effects of BTR were reported in marine fish. Although not much is known about the toxicity of these compunds, these reasons were sufficient for some countries like Australia and Germany had established a maximum limit of 7 ng/L in the drinking water guidelines for the tolyltriazole (which is a mixture of the isomers 4TTR and 5TTR) (11,13,16,20).



(a) (b)

Figure 2 - Molecular structure of the benzotriazoles studied in this work

(a) benzotriazole, (b) 5-methylbenzotriazole

1.3 Benzothiazoles

Benzothiazoles are aromatic heterocyclic compounds formed by a 1,3-thiazole ring fused with a benzene ring, and the chemical formula of the parent compound is C_7H_5NS (11). Benzothiazoles are high production volume emerging environmental pollutants, and its production was reported to be in the range of 4.5 to 450 tons in the United States of America in 1993 (20,21). Some of the most commonly known benzothiazoles include benzothiazole (BT), 2-hydroxybenzothiazole (HOBT) and 2-(methylthio)benzothiazole (MeSBT), which are the substances of this group of compounds that are going to be tested in this study (Figure 3). BT and its derivative are commonly used in the production of rubber and as herbicides, fungicides (in paper and leather industries), photosensitizers in photography and corrosion inhibitors. They also have applications in drugs, de-icing fluids and food flavours, for example. BT core is highly important scaffold for drug development because it has demonstrated a wide spectrum of pharmacological activities such as anticancer, anti-inflammatory, antimicrobial and antidiabetic (19,20,22–24).

Despite its widespread use and environmental occurrence, little is known about the toxicity of these compounds. BT derivatives were reported as endocrine-disrupting in laboratory animals, dermal sensitizers, respiratory tract irritants and were linked to mutagenicity in microorganisms and carcinogenicity in humans (16,20,22).

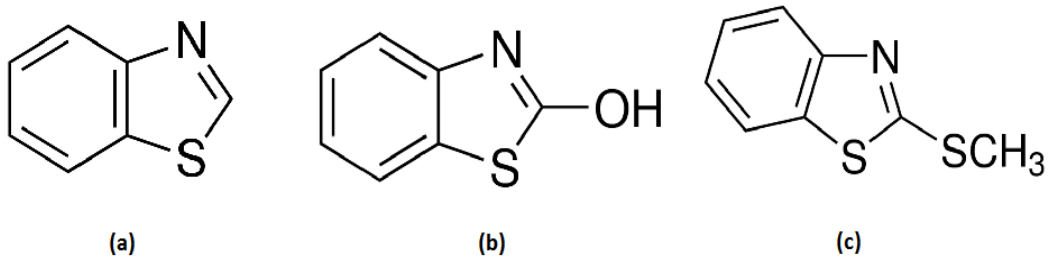


Figure 3 - Molecular structure of the benzothiazoles studied in this work

(a) benzothiazole, (b) 2-hydroxybenzothiazole, (c) 2-(methylthio)benzothiazole

1.4 Microalgae as biosensors

There are two types of methodologies that can be used to evaluate the water quality: the chemical analysis and the biosensors. The "chemical sensors" (defined as a device that transforms chemical information into an analytical signal) have the advantages of giving results that are highly reproducible, precise and that can detect specific chemicals at low concentration levels, however, they are characterized for being costly, time-consuming, requiring a pre-treatment of the sample and are limited to a restricted number of species (4,6,25).

Commonly, the emerging organic water pollutants tested in this work (BSA, BT and BTR and its derivatives) like other organic water contaminants, are analysed, after extraction, by chemical methods like liquid chromatography or gas chromatography coupled preferably with tandem mass spectrometry (11). In this work we will use biosensors as the analytical method of analysis. A biosensor can be defined as a combination of a bioreceptor or bioindicator, the biological component, and a transducer, the detection method. The bioreceptor will identify the analytes and create a biological response in its presence and the transducer will transform the biological event into an electrical signal that can be measured electrochemically, optically, acoustically, mechanically, calorimetrically or electronically and this measurable signal will be proportional to the concentration of that analytes (4,25,26).

Since the first biosensor was created, in 1962, that they have been intensively studied and used in many different applications. Biosensors have the advantages of having high sensitivity, simplicity or unnecessary sample pre-treatment, quick

responses, lower costs and there is the possibility of being permanently settled in the areas under control to give continuous on-site detection and analysis (4–6,25,26). Many bioassays using bacteria, plant tissues, animal cells and microalgae have been developed in the last decades (27).

In this work we used microalgae as biosensors. Microalgae are microscopic photosynthetic organisms that are ubiquitous in every aquatic environment and there are many reasons why algal indicators should be used for toxicology research and environmental risk assessment of chemicals: they are very sensitive and are at the beginning of the trophic chain so they represent a good biological marker and an early warning system of the pollution in the ecosystem. Microalgae bioindicators also have short response times, reproducibility and require few pre-treatment of the samples which makes it possible to use them on the field to detect environmental contaminants. One of the limitations of this kind of biosensors is their low specificity: the biological response of the microalgae can be a synergic response to a several number of contaminants to which the microalga is sensitive to (4,25,28).

The contaminants can induce toxicity in the microalgae by several different ways. There is the possibility that the simple contact of the contaminant with the cell surface could be enough to stimulate the production of ROS and subsequent lipid peroxidation, shortage of metabolic energy and decline of photosynthetic energy. Some contaminants are capable of disrupting cell's organelles, components and/or molecular structure leading to a cascade of events where the cell tries to compensate the effect of the damaging factors, but when the damaging factor is greater or more prolonged exposure, violation of cellular functions occurs. For example, some pollutants can disturb the balance between the oxidant and antioxidant systems, changing the expression of genes related to photosynthesis, glycolysis, fatty acid biosynthesis and β-oxidation and others, by incrementing the ROS production inside the microalgae cell, free radicals are generated which induces DNA damage and with the loss of the cell ability to detoxify it leads to the cell death and the reduction or complete inhibition of cell growth due to chromosome instability and mitosis inhibition (29–34)

Understanding the many different ways by which the contaminants induce toxicity in the microalgae, is easy to understand that there will be different methods to evaluate that toxicity. Usually, toxicity assays using microalga as biosensors, involve

one or more of these techniques: measuring the chlorophyll fluorescence emission, performing growth inhibition assays, evaluating the oxidative stress (by measuring the production of reactive oxygen species (ROS), the activity of antioxidant enzymes like superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase or by evaluating the lipid peroxidation by testing the levels of malondialdehyde). We can also directly measure the content of chlorophyll extracted from the microalga, determine the lipids and fatty acid concentrations in the microalga culture or evaluate the effects of the substance on transcription of genes related to photosynthesis, glycolysis, fatty acid biosynthesis and β-oxidation, for example (4,30–32). In this work, we will only perform growth inhibition assays to evaluate the toxicity of the emerging pollutants in study.

1.4.1 *Dunaliella tertiolecta*

Dunaliella species are unicellular and mobile biflagellate microalgae that belong to the phylum clorophyta. This green marine microalga *Dunaliella tertiolecta*, like the other microalgae of the *Dunalliela* species, is morphologically characterized by the lack of a cell wall and being coated with a mucilaginous envelope (Figure 4) (32,35).

This microalga fulfils most of the criteria for an ideal bioindicator: it is easy to cultivate in a laboratory and has a rapid growth and response to environmental pollutants. These characteristics combined with its ability to grow in severe conditions and the lack of a rigid cell wall (eliminating a potential barrier to the permeation of the contaminants tested) makes *Dunaliella tertiolecta* one of the best microalgae to use as a bioindicator for the evaluation of environmental contamination (35,36).

1.4.2 *Phaeodactylum tricornutum*

Diatoms are the most species-rich group of algae in the marine environment. *Phaeodactylum tricornutum* is a diatom with a rigid siliceous cell wall (less permeable than the cell wall of the green algae) that is present in transitional, marine-coastal and marine waters and is the only really standardized marine algae species for waste water toxicity tests (Figure 4) (32,36–39).

Its use as a standard marine species in bioassays on the toxicity of chemical pollutants is due to its easy cultivation, significant sensitivity to environmental

pollutants, is ecologically well-defined and its morphology and genome is already well known (30,31,39).

1.4.3 *Pseudokirchneriella subcapitata*

As a representative of the freshwater environment in this work we used the freshwater algae *Pseudokirchneriella subcapitata* also known as *Selenastrum capricornutum* or *Raphidocelis subcapitata*. *P.subcapitata* is a green alga (clorophyta), unicellular, non-motile, and sickle-shaped that is normally found in unicellular form (Figure 4). Because of its high growth rate, easy to cultivate in laboratory, being representative of eutrophic and oligotrophic fresh water environments and high sensitivity to a very different number of substances makes this green algae one of the most frequently used organisms in toxicity studies (9,40,41).

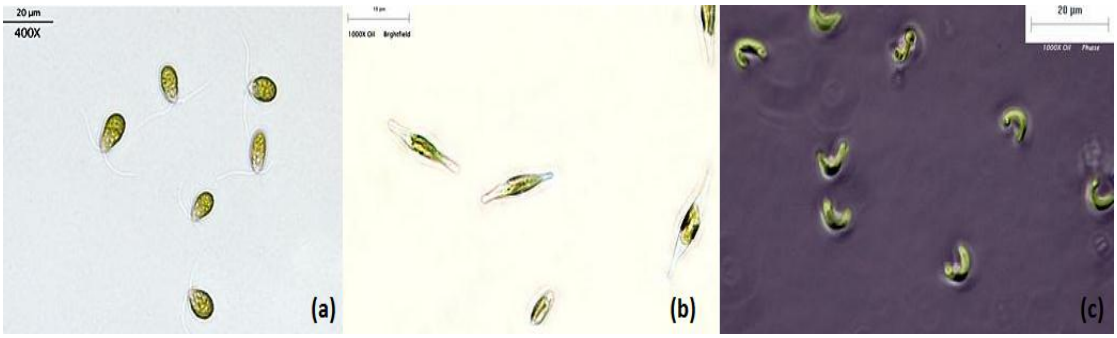


Figure 4 – Microscopic visualisation of the three microalgae used in this study

a) *Dunaliella tertiolecta*, b) *Phaeodactylum tricornutum*, c) *Pseudokirchneriella subcapitata* (42)

1.5 Spectrophotometry

Among various techniques, colometric sensing techniques are one of the most widely used in the development of microbial sensors mainly due to its simplicity and low cost. The colour alterations can be distinguished by the naked eye or by a spectrophotometer. The advantage of using a spectrophotometer and not just macroscopic observation is that some colometric differences are not distinguishable by the naked eye but its absorbance can be easily measured at a certain wavelength and the measured signal can then be correlated with the analyte concentration. Growth inhibition assays are one of the most used methods to assess some substances toxicity to some microalgae used as biosensors and they are performed using a spectrophotometer, which correlates microalgae density to light absorbance at a

specific wavelength. Light absorbed by chlorophyll is the base for spectrophotometry use for microalgae density assessment (26,43).

A spectrophotometer is the absorbance instrument used to collect ultraviolet-visible spectra, which will measure the amount of light absorbed by the sample. When monochromatic light passes through a solution there is a quantitative relationship between the solute concentration and the intensity of the transmitted light, in a relationship explained by the Beer-Lambert law, which demonstrates that the more light the coloured sample absorbs the higher the absorbance value and the lower the amount of radiation that crosses the sample. Absorbance is equivalent to optical density or light extinction and is directly proportional to the concentration of the coloured compound (44).

There are three types of spectrophotometers (single beam, double beam and simultaneous spectrophotometer) and they all include a light source (that produces the light that will pass through the filter), a sample holder and a detector (that will measure the light that passed through the sample cuvette and translate it into an absorbance value that can be easily read) but some have a filter (or monochromator) for selecting one wavelength at a time. In this work we will use a double beam spectrophotometer (Figure 5) which has a single light source and a monochromator and then there is a splitter and a series of mirrors to get the light beam to a reference sample and the sample to be analysed which allows more accurate readings (this equipment will measure the sample and control simultaneously, automatically subtracting the control signal from the sample signal, presenting the sample's real absorbance value). Working in the visible region of the electromagnetic spectrum, sample cuvettes can be made of glass or plastic (absorbance readings between 380 and 780nm) (44).

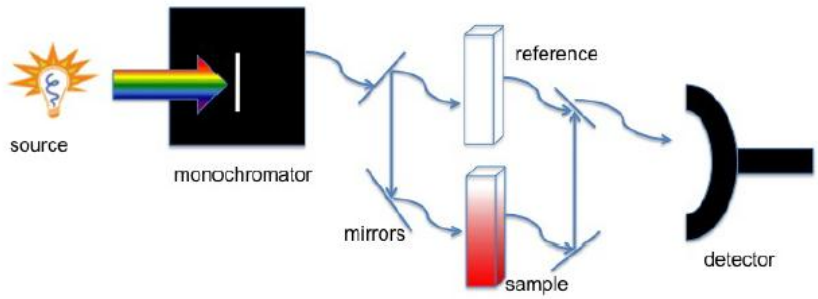


Figure 5 – Illustration of a double-beam spectrophotometer (44)

2 Materials and methods

2.1 Equipment

- Autoclave Vapormatic 770, EMC™
- White lamp/red lamp Osram daylight 2 x 36W + Osram Gro-Lux 36W
- Double-beam UV/Vis spectrophotometer from Jasco™, model 7800
- Optical microscope NIKON™ Eclipse E400
- Bürker counting chamber

2.2 Materials

Besides the material commonly used in the laboratory, specific material used is listed in this section:

- Plastic test tubes
- Plastic spectrophotometer curvettes
- Cotton and gauze cover
- 250mL Erlenmeyer flasks
- 500mL glass bottles

2.3 Microalgae

- Microalgae *Dunaliella tertiolecta,, Phaeodactylum tricornutum* and *Pseudokirchneriella subcapitata* were offered to the labboratory by the Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale", Teramo, Italy

2.4 Reagents

All chemical reagents used were of analytical grade or superior.

- The pollutants in test **(**benzenesulfonamide, *para*-toluenesulfonamide, benzothiazole, 2-hydroxybenzothiazole, 2-(methylthio)benzothiazole,

benzotriazole, and 5-methylbenzotriazole) were directly bought from Sigma-Aldrich®

- Synthetic sea salt (Instant Ocean®, aquarium systems)
- Deionised purified water (obtained in the laboratory using Millipore equipment)
- Formaldehyde (Fluka®)
- Alga *Pseudokirchneriella subcapitata* nutrients (bought directly from Sigma-Aldrich®)
    1. Calcium nitrate tetrahydrated – $Ca(NO_3)_2 .4H_2O$
    2. Monopotassium phosphate – KH_2PO_4
    3. Magnesium sulphate heptahydrated – $MgSO_4.7H_2O$
    4. Sodium bicarbonate – $NaHCO_3$
    5. Ethylenediaminetetraacetic acid ferric sodium – EDTAFeNa
    6. Boric acid (H_3BO_3) + Ammonium molybdate tatrahydrated [$(NH_4)_6Mo_7O_{24}.4H_2O$] + Manganese(II) chloride tetrahydrated ($MnCl_2.4H_2O$)
    7. Cyanocobalamin + Biotin + Thiamine
    8. Sodium nitrate – $NaNO_3$
    9. Sodium dihydrogen phosphate dihydrated – $NaH_2PO_4. 2H_2O$
- Algae *Dunaliella tertiolecta* and *Phaeodactylum tricornutum* nutrients (bought directly from Sigma-Aldrich®)
    1. Ethylenediaminetetraacetic acid disodium ($EDTANa_2$) + Iron chloride hexahydrated ($FeCl_3.6H_2O$) + Copper sulphate pentahydrated ($CuSO_4.5H_2O$) + Zinc sulphate heptahydrated ($ZnSO_4.7H_2O$) + Cobalt(II) chloride hexahydrated ($CoCl_2.6H_2O$) + Manganese(II) chloride tetrahydrated ($MnCl_2.4H_2O$) + Sodium molybdate dihydrated ($Na_2MoO_4.2H_2O$)
    2. Cyanocobalamin + Biotin + Thiamine
    3. Sodium silicate – Na_2SiO_3
    4. Sodium nitrate – $NaNO_3$
    5. Sodium dihydrogen phosphate dihydrated – $NaH_2PO_4.2H_2O$

2.5 Experimental procedures

2.5.1 Microalgae cultures

To prepare the culture medium for the K microalga we used sterilized deionised water. To 100mL of sterilized water, 100µL of each of the nine nutrients were added in order to achieve the final culture medium for this microalga.

For the marine microalgae, Ph and D, synthetic marine water was prepared by adding 16.5g of Instant Ocean® salt to 500mL of deionised water. For the Ph microalga, the culture medium was made by adding 100µL of nutrients 1, 3, 4 and 5 and 10µL of the nutrient 2 to 100mL of sterilized synthetic sea water. The medium for the D microalga was prepared by adding 100µL of nutrients 1, 3 and 4 and 10µL of nutrient 2 to 100mL of sterilized synthetic sea water.

The starter culture of each microalga was made by inoculating 1mL of a previous starter culture on a 250mL Erlenmeyer flask containing 100mL of the appropriate culture medium previously prepared. The flasks were covered with a sterilized and porous cotton and gauze cover and maintained under continuous white illumination in the culture chamber at a room temperature around 20ºC (Figure 6). New starter cultures were prepared every month.

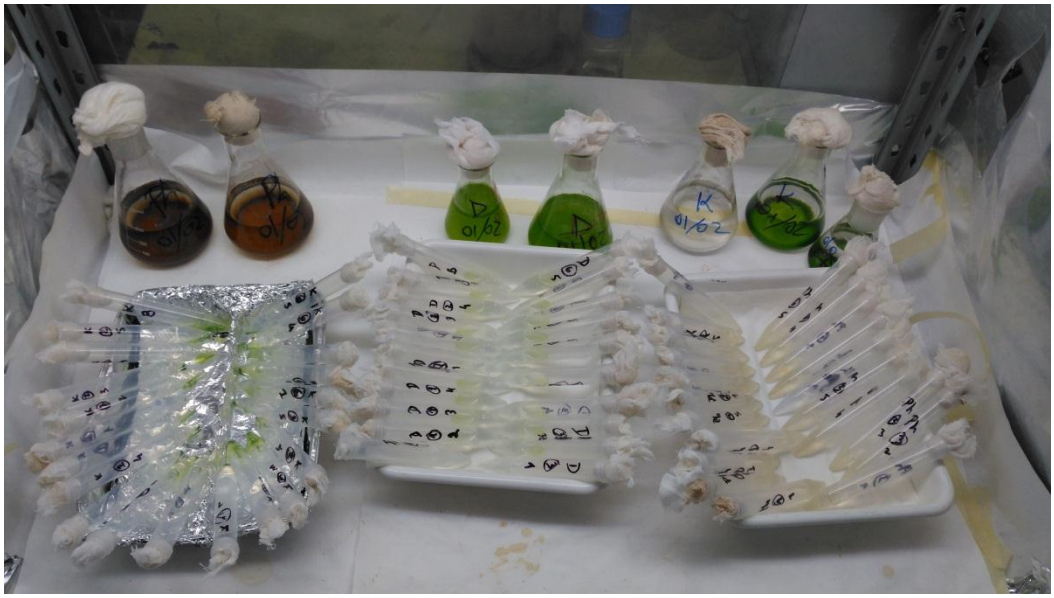


Figure 6 – Microalgae culture chamber in the laboratory.

Samples and control tubes are in the front and starter cultures in the back.

2.5.2 Samples and controls solutions

Each sample occupied one test tube and comprised the starter culture of the alga in study, the pollutant and growth medium. Each control solution occupied one test tube as well and comprised only the alga in study and the growth medium.

For each test one Erlenmeyer flask was filled with 200mL of sterilized deionised water or synthetic marine water, depending on the alga in study, the respective specific nutrients as described above and 2mL of the starter culture prepared before.

For the first test of each pollutant studied for each alga the tubes were filled with 5mL of the solution prepared (alga starter culture + growth medium) but for the second test we decided to change it to 10mL since that on the first tests some tubes reached 4mL of total volume due to the water evaporation throughout the test and we felt the need to fill it again with 1mL of sterilized deionised water to avoid that the concentration of the medium could affect the microalga growth which led to the dilution of the medium and consequent lower absorbance values.

With the 10mL tubes we verified that some tubes almost reached 9mL of total volume by the end of the test (although none of them actually reached 9mL) which means that with this volume we only concentrated the medium in less than 10% which wouldn't be sufficient to affect the growth of the microalga, when compared to the 20% on the first test that is a much more significant percentage.

Six test tubes were filled with 5mL of the solution prepared in the Erlenmeyer flask on the first test, and with 10mL on the second test. One of the tubes was marked as control and to the other five tubes the volume of the pollutant correspondent to the final concentration desired (100ppm, 50ppm, 25ppm, 10ppm, 5ppm, 1ppm, 0.1ppm or 0.01ppm) was added and these five tubes were marked as samples and with the number corresponding to the pollutant concentration. The six tubes were then covered with sterilized porous cotton and gauze covers and kept under the same conditions as the starter cultures (under continuous illumination of a white lamp, at a room temperature around 20ºC and with daily monitoring) (Figure 6).

2.5.3 Growth inhibition assays

Growth inhibition assays were made using spectrophotometry and macroscopic observation. Absorbance measurements were made every week, in order to obtain three absorbance values where, preferably, the first one in the beginning of

the microalga growth, the second one during the exponential phase and the last one with a high cell density inside the tube. The first measurement was made at the first macroscopic sign of growth, which happened when the control showed some perceptible green/brown coloration inside the tube.

The absorbance measurements were made at 545.6nm, which was the wavelength at which the absorbance peaks occurred.

Samples and controls were analysed without dilution and before the absorbance measurements the equipment was calibrated using the correspondent standard. Clean plastic cuvettes were filled with the sample or the control and after each reading the solution was replaced into its original test tube.

2.5.4 Cell counting

Whenever necessary, microalgae cells were counted, using a Bürker counting chamber and an optical microscope. For the D microalgae cells, due to their mobility, 50µL of formaldehyde were added to 200µL of sample in order to immobilize the cells and allow their counting.

2.5.5 Data analysis

The programme used for data analysis was Microsoft Office Excel. Line chart graphics were fitted directly on the data registered, for the spectrophotometric analysis of growth inhibition.

EC_{50} values were estimated from the comparison of the registered value for the control sample with all other values obtained from the other curves, at the last point of measurement. Calculating half the value obtained for the control graphic at the last point, EC_{50} value was estimated based on the two concentration curves closest to the obtained value for the variable.

3 Results

Growth inhibition assays for each microalgae were performed using different concentrations of the seven different water pollutants tested (benzenesulfonamide, benzothiazole, benzotriazole, *para*-toluenesulfonamide, 2-hydroxybenzothiazole, 2-(methylthio)benzothiazole, 5-methylbenzotriazole).

When we were able to observe, macroscopically, some coloration (brown for the *Phaeodactylum tricornutum* and green for the *Dunaliella tertiolecta* and *Pseudokirchneriella subcapitata*) in the control tube, indicating that the microalgae had grown, we proceeded with the first measurement and after that two other measurements every week (preferably after seven days) with a total of three measurements for each tube.

For unknown reasons, the microalgae took more time to start growing than we predicted and also had variations concerning the first time we measured their growth since some of the control tubes showed a perceptible coloration after 14 days and some of the other control tubes only after 25 days.

After the three measurements we evaluated the results and repeated the procedure with the same range of concentrations or a different range depending on the results for the sensitivity of the microalgae to the first one (the K microalga appeared to be more sensitive to lower concentrations of the emerging pollutants on the first tests so we decided to repeat the test with the same range of concentrations – 100ppm to 0.01ppm – only for this microalgae, and for the Ph and D microalgae on the second test we decided to change it to 100ppm to 5ppm).

As mentioned before, these pollutants usually occur in natural aquatic environments with concentrations that can go up to hundreds of µg/L (or ppb). So for the first range of concentrations we decided to choose as the minimum concentration of the pollutants of 10 ppb (or 0.01ppm) and the maximum of 100 ppm and depending

on the sensitivity of the microalgae and the toxicity of these contaminants we chose the same or different range of concentrations for the second batch of tests.

3.1 Benzenesulfonamides toxicity

3.1.1 Benzenesulfonamide

3.1.1.1 *Dunaliella tertiolecta*

The D microalgae took 25 days until we consider that the coloration in the control tube was perceptible and that we could proceed with the first measurement. On the 25th day we could macroscopically observe that there was a clear difference between the tube containing 100ppm of BSA (which looked limpid) and the other tubes, which seemed similar to each other.

After reading the absorbance on the spectrophotometer on the 25th, 28th and 33th of culture we could conclude that the D microalga growth was clearly affected with the concentration of the BSA of 100ppm (as we could see macroscopically) and slightly affected with 10ppm of BSA on the tube, but for concentrations under 10ppm the growth rate was not affected, since it was similar to the control (Figure 7).

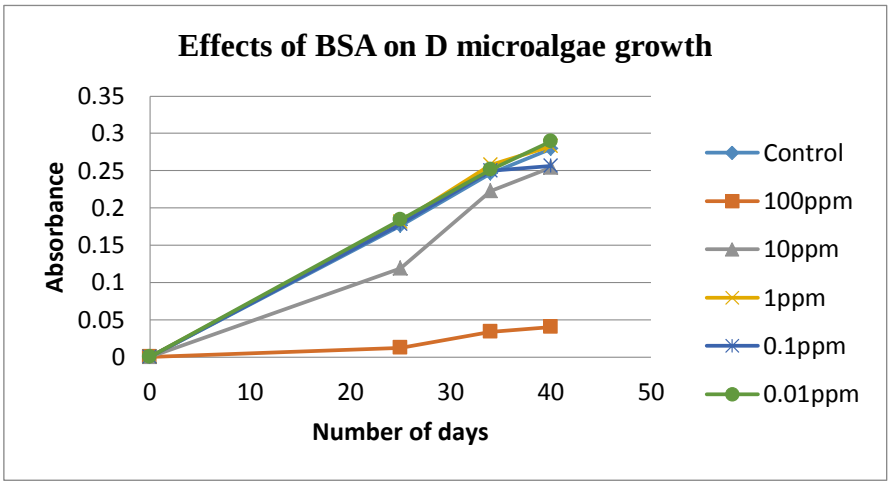


Figure 7 – First test of microalga D growth rates with different concentrations of BSA in the medium

On the second test, we could see a clear coloration on the tubes on the 20th day unlike the first batch that we had to wait till the 25th day to proceed with the first absorbance measurement. On the naked eye we could see the different coloration intensity on the tubes: the tubes with concentrations of 100ppm, 50ppm, and 25ppm of BSA had a lighter green colour when compared to the other tubes.

The absorbance values, read on the 20th, 28th and 33th days, were consistent with the conclusions taken macroscopically: the D microalgae growth is affected when there is BSA in the medium if the concentrations are equal to 25ppm or higher (Figure 8).

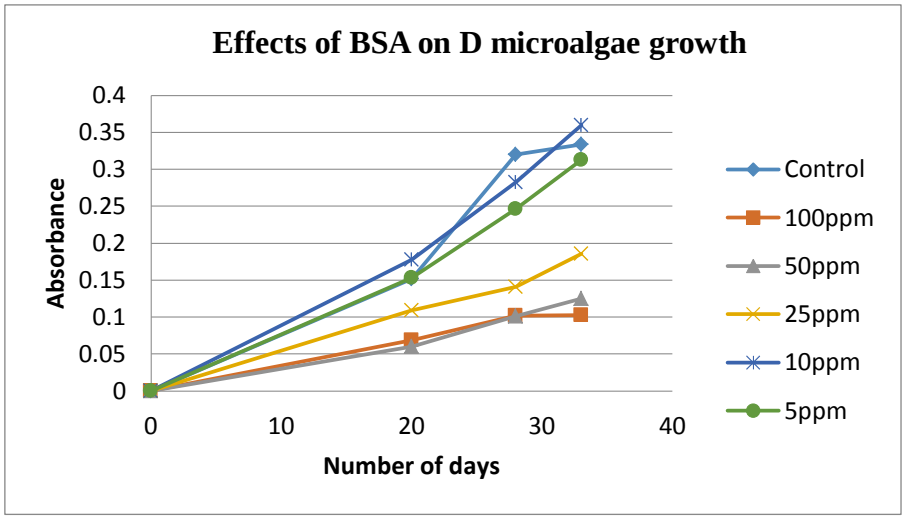


Figure 8 – Second test of microalga D growth rates with different concentrations of BSA in the medium

Comparing the results of the two test we can conclude that the D microalgae growth is affected by the presence of BSA and its growth is lower when the concentrations of this contaminant is equal to or higher than 25ppm in the water. The EC_{50} estimated is between 25ppm and 10ppm.

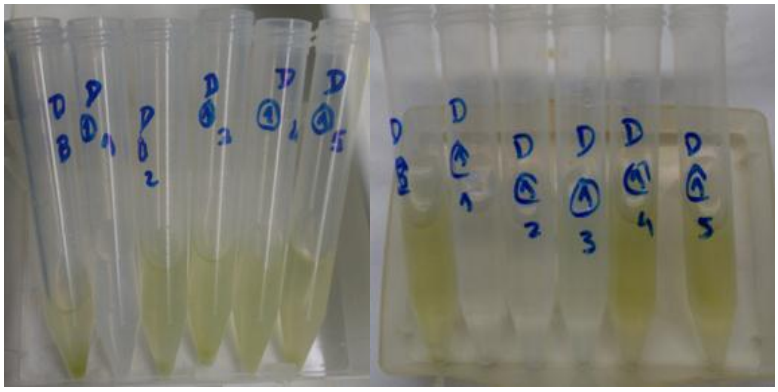


Figure 9 – Samples and control tubes of the first test (left) and second test (right) of the D microalga culture with BSA on the last day of the test

3.1.1.2 *Pseudokirchneriella subcapitata*

For the K microalga we could see a perceptible green colour in the control tube on the 15th day, so we chose to proceed with the first measurement.

The absorbance values, especially on the 21st and 26th days, showed what we observed macroscopically: the microalgae growth was affected by concentrations of 100ppm, 1ppm, 0.1ppm and 0.01ppm but it was not affected by the concentration of 10ppm, which didn't allow us to take any conclusions due to the fact that if the growth wasn't affected with 10ppm of BSA, it shouldn't be affected by lower concentrations than 10ppm (Figure 10).

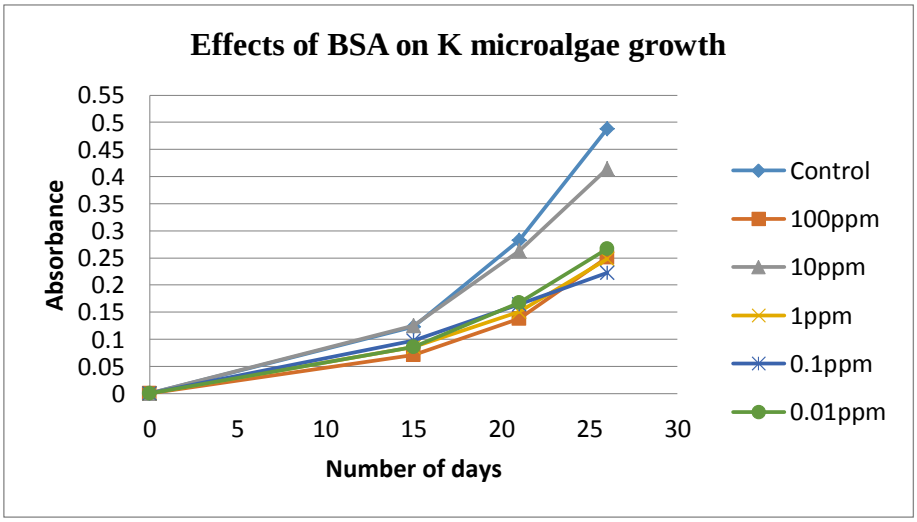


Figure 10 – First test of microalga K growth rates with different concentrations of BSA in the medium

For the second batch of tubes, although we couldn't macroscopically see a very clear green coloration on the 21st day of culture, we decided to evaluate the growth of the microalgae on the spectrophotometer, so that the day of first measurement would be as close as possible to the one on the first test. We could conclude that the BSA didn't affect the K microalga growth since the absorbance values obtained on the three different culture days were very close to the control tube (Figure 11).

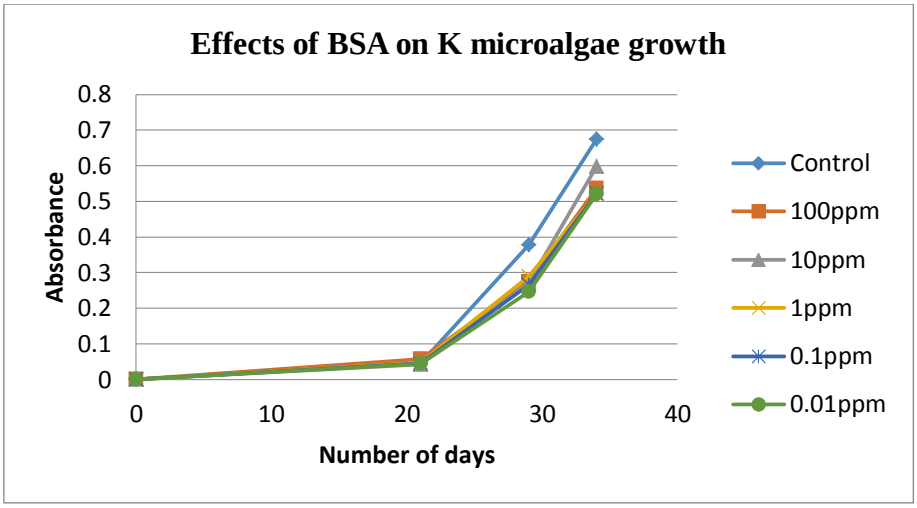


Figure 11 – Second test of microalga K growth rates with different concentrations of BSA in the medium

Although on the first test some concentrations of BSA affected the K microalga growth, the results didn't make sense since 10ppm of BSA in the medium didn't affect the growth but lower concentrations did: these results combined with the fact that the first test wasn't consistent with the second test (the microalgae growth wasn't affected by any concentration of BSA) doesn't make it possible to take any conclusions about the sensitivity of the K microalgae to the BSA or if the presence of this contaminant in the water affects the growth of the K microalgae, so further studies should be performed.

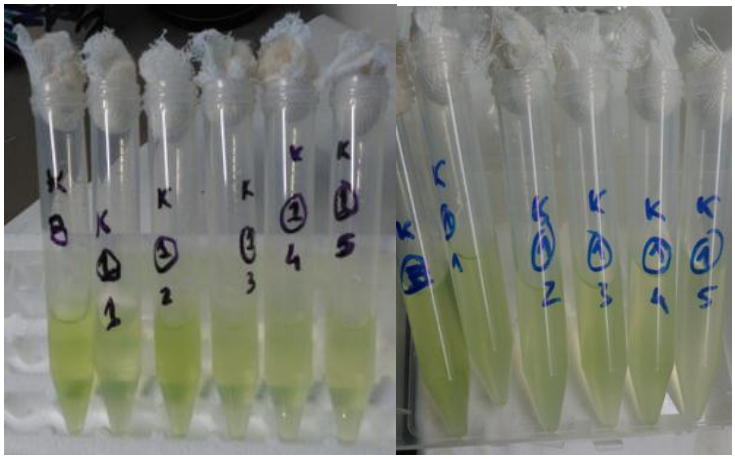


Figure 12 – Samples and control tubes of the first test (left) and second test (right) of the K microalga culture with BSA on the last day of the test

3.1.1.3 *Phaeodactylum tricornutum*

On the 15th day the brown coloration was more perceptible and the intensity of the brown colour was similar to all the tubes, which was consistent with the absorbance results. On the 21st day of culture the results were like the previous ones, showing that the presence of BSA on the medium didn't affect the Ph microalgae growth. After the absorbance measurements on the 21st day we noticed that the total medium volume was about 4 mL, which was 1 mL lower than the initial volume (5 mL) due to the evaporation of the water that indicated that the medium was more concentrated, which could affect the final results. In order to prevent that the results could be affected by the concentration of the medium, 1 mL of purified water was added to each tube. This explain the lower absorbance values on the 26th day however, we can also conclude with the results of this final measurement that the growth was not affected by the presence of BSA in the medium since the absorbance values were all close to the control tube (Figure 13).

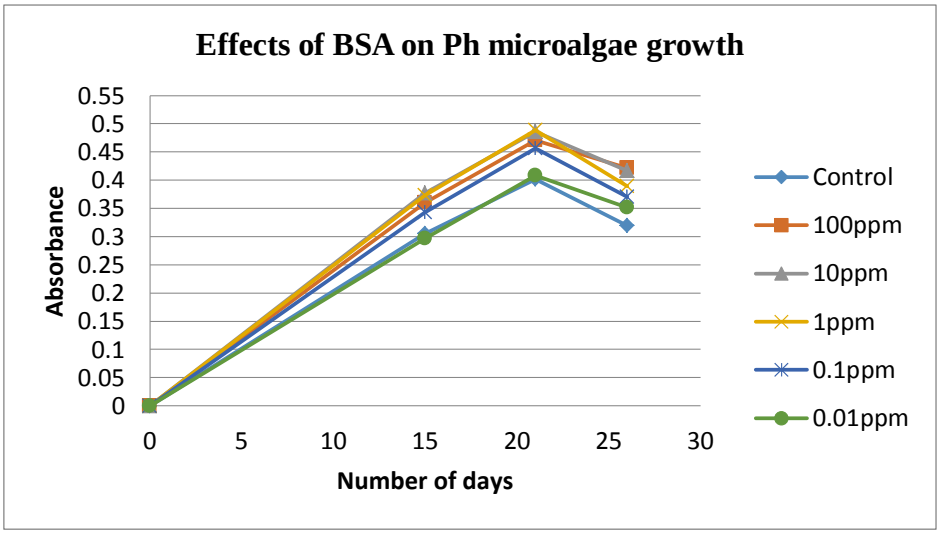


Figure 13 – First test of microalga Ph growth rates with different concentrations of BSA in the medium

On the second test with a different range of concentrations and a total volume of 10mL the growth of the Ph microalga was similar in all the tubes, with and without BSA in the medium, which was consistent with the results on the first test (Figure 14).

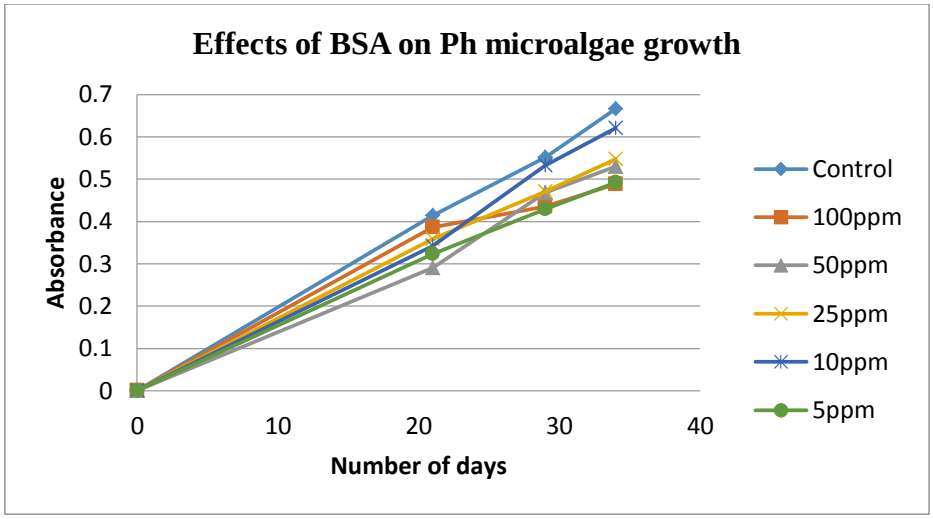


Figure 14 – Second test of microalga Ph growth rates with different concentrations of BSA in the medium

Since that on both tests the BSA didn't affect the growth of the Ph microalga we can conclude that this microalga is not sensitive to the presence of this contaminant in the water, for concentrations of 100ppm or lower.

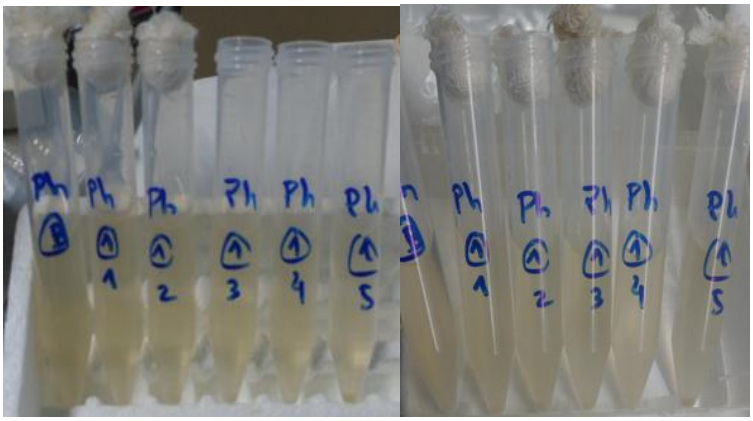


Figure 15 – Samples and control tubes of the first test (left) and second test (right) of the Ph microalga culture with BSA on the last day of the test

3.1.2 *para*-toluenesulfonamide

3.1.2.1 *Dunaliella tertiolecta*

To evaluate the effects of the pTSA on the growth of the D microalga we started measuring the absorbance of the six tubes on the 25th day of culture and the results obtained by spectrophotometry showed that every tube, except for the tube with the lower concentration of pTSA (0.01ppm), had lower absorbance values than the control sample. These results didn't differ from the ones obtained on the 32nd and 39th day of culture, with the slight difference that for the higher concentrations of the

pollutant (100ppm and 10ppm) the absorbance values for these tubes were even more distant than the values for the other samples (Figure 16).

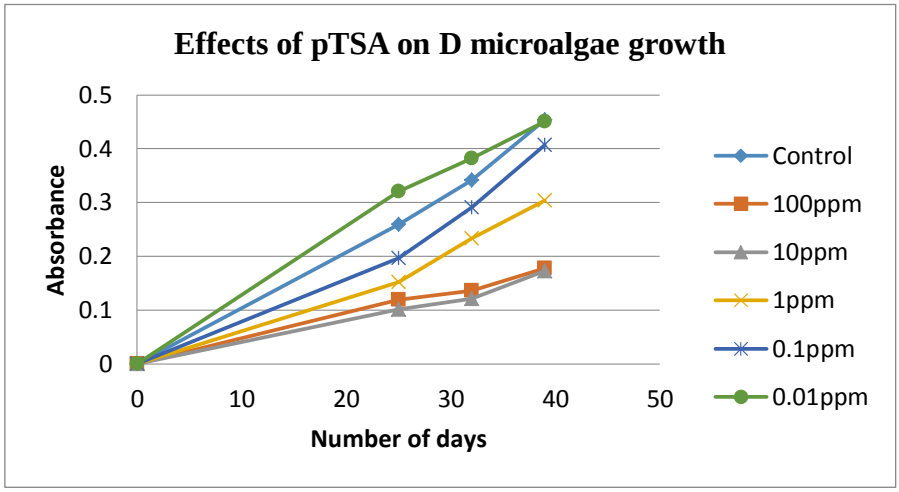


Figure 16 – First test of microalga D growth rates with different concentrations of pTSA in the medium

On the second test, both macroscopically and by spectrophotometry the pTSA seemed to have an influence on the growth of the D microalga on the three measurements (14th, 22nd and 29th days of culture) and the absorbance values were similar to each other for the whole range of concentrations and significantly lower to the values of the control (although the sample with 50ppm of pTSA had a higher value than the others, when it should only be higher than the one with 100ppm of the pollutant, however it was still much lower than the control, and its higher value only happened on the last measurement) (Figure 17).

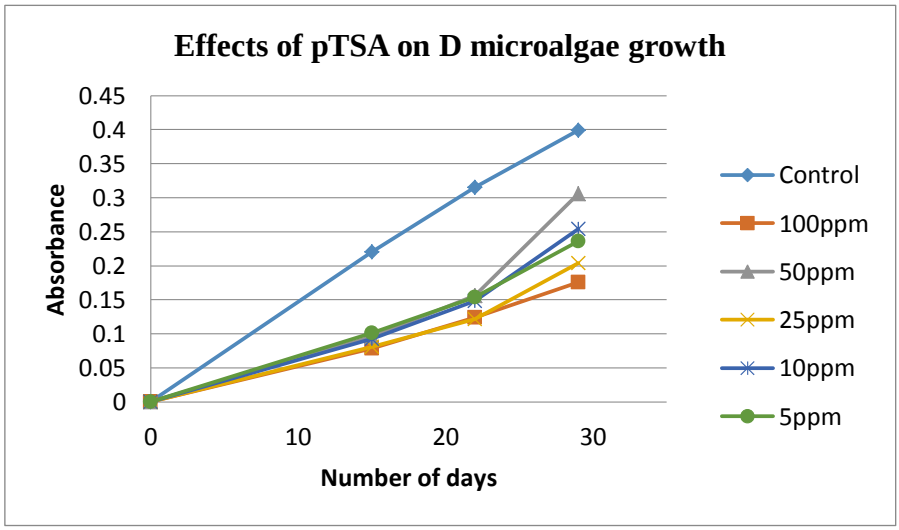


Figure 17 – Second test of microalga D growth rates with different concentrations of pTSA in the medium

We can conclude by the results of both tests that the growth of the D microalga is affected by the presence of pTSA in the water, when its concentration is higher or equal to 1ppm (although on the first test the absorbance values of the 0.1ppm sample were lower than the control, they weren't as significantly lower as the other samples – with a reduction on the growth rate of the of less than 10% on the last measurement). The EC_{50} of this pollutant for the D microalga was estimated to be between 25ppm and 10ppm.

Figure 18 – Samples and control tubes of the first test (left) and second test (right) of the D microalga culture with pTSA on the 32nd and 22nd days, respectively

3.1.2.2 *Pseudokirchneriella subcapitata*

For the first test to evaluate the effects of the pTSA on the K microalga growth we decided to do the first spectrophotometric measurement on the 15th day of culture. Although macroscopically there was no perceptible green coloration on the tubes, all the other first tests for the K microalga concerning other pollutants were evaluated on the 14th or 15th day so we decided not to wait for a macroscopic evidence of growth and proceed with the first measurement. As expected, the results were inconclusive since that the absorbance values were very low and all very close to each other and to the control.

The absorbance values on the 20th and 31st days of culture allowed us to take the same conclusions as we did macroscopically: the growth of the K microalga was affected only by the presence of 100ppm of pTSA in the medium. On the 31st day we can see that the concentrations of 10ppm and 1ppm also reduced the growth of the K microalga however this only seemed to happen at the end of the test and in a much lower level than the 100ppm concentration (Figure 19).

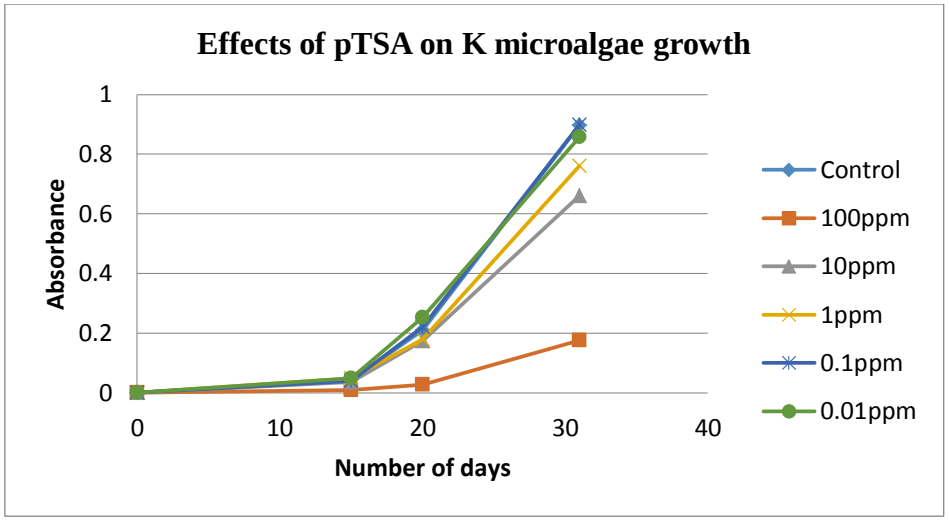


Figure 19 – First test of microalga K growth rates with different concentrations of pTSA in the medium

On the second test the results were very similar to the ones on the first test with only a few exceptions: macroscopically the intensity of green coloration of the tube containing a concentration of 100ppm of pTSA was closer to the control and so were the absorbance values. That being said the same concentration of pTSA in the medium (100ppm) didn't affect the growth of the microalga as much as on the first test however the difference between the growth rate of the control and the 100ppm sample was clear on both tests. On this second test, like on the first one, the K microalga growth was also slightly affected by the presence of 10ppm in the medium (only perceptible on the last measurement also) but unlike the first test the concentration of 1ppm didn't affect its growth this time (Figure 20).

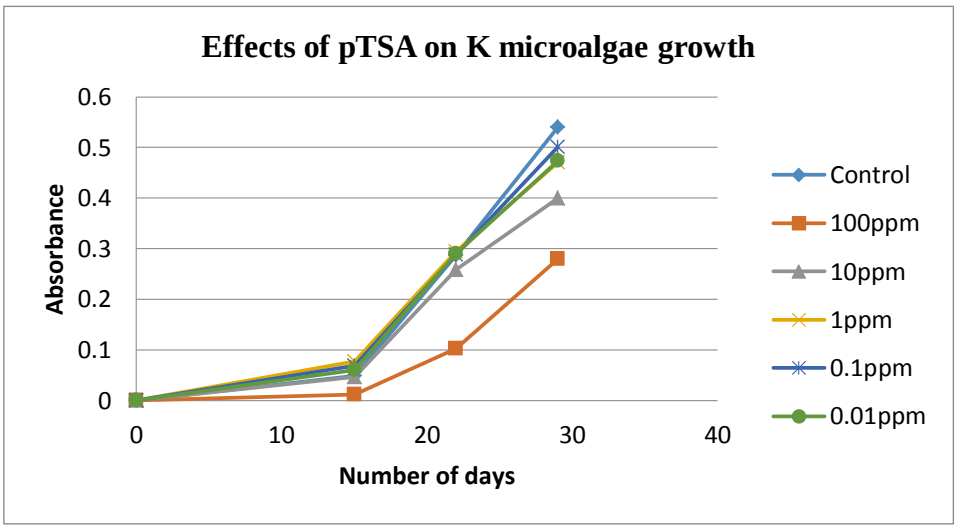


Figure 20 – Second test of microalga K growth rates with different concentrations of pTSA in the medium

With the results of both tests we can conclude that the K microalgae are sensitive to concentrations of pTSA equal or higher than 10ppm (the sensitivity of the K microalga to concentrations between 10ppm and 100ppm should be evaluated on further studies anyway in order to try to understand why 10ppm of pTSA in the medium only affected the growth by the end of the study). The EC_{50} estimated is between 100ppm and 10ppm.

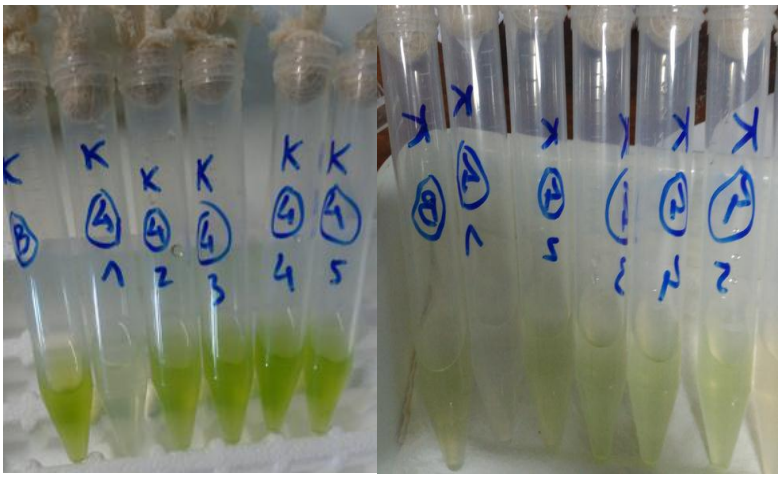


Figure 21 – Samples and control tubes of the first test (left) and second test (right) of the K microalga culture with pTSA on the last day of the test and on the 22nd day, respetively

3.1.2.3 *Phaeodactylum tricornutum*

On this first test, we read the absorbance of the samples on the 14th, 19th and 30th days of culture and the results were unexpected: on the 14th day the absorbance results were low so it didn't allow us to take any definite conclusions; on the 20th every sample had a similar absorbance value and on the 30th all the samples had a significantly higher absorbance value than the control (for unknown reasons) except for the culture with 100ppm of pTSA in the medium but not significantly lower as we would want in order to consider that the growth was clearly reduced (Figure 22).

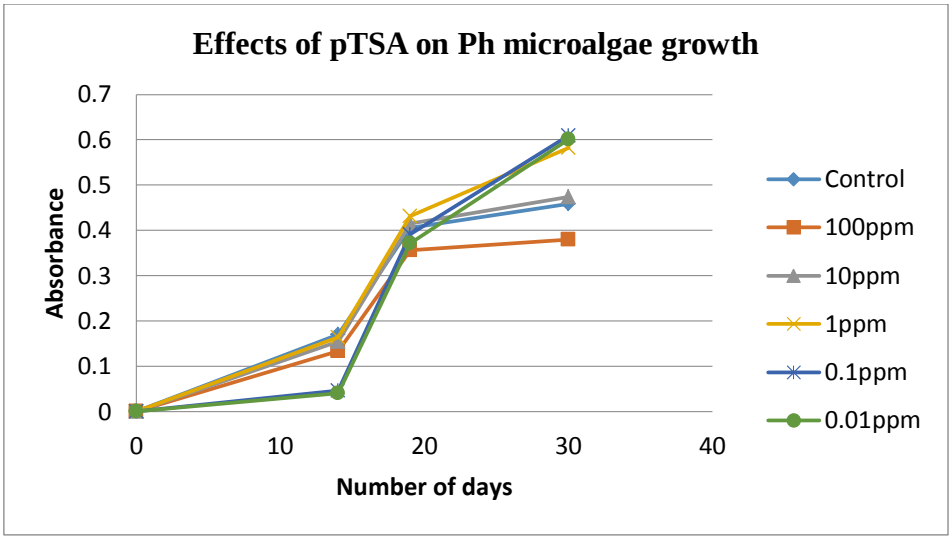


Figure 22 – First test of microalga Ph growth rates with different concentrations of pTSA in the medium

On the second test, with a different range of concentrations, in all the three measurements done (on the 15th, 22th and 29th days of culture) all the absorbance results were lower than the control but they were all similar to each other and not as significantly lower than the control as necessary to consider that the growth was with no doubts affected by the presence of pTSA in the medium (Figure 23).

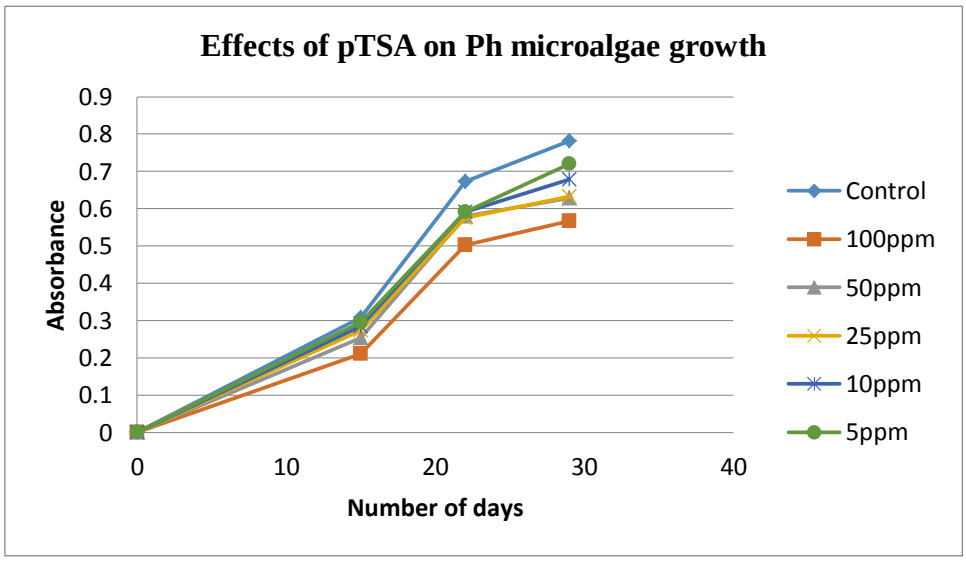


Figure 23 – Second test of microalga Ph growth rates with different concentrations of pTSA in the medium

The comparison of the results on both tests allowed us to conclude, that concentrations of pTSA equal or lower than 100ppm in the water doesn't affect the Ph microalgae growth.

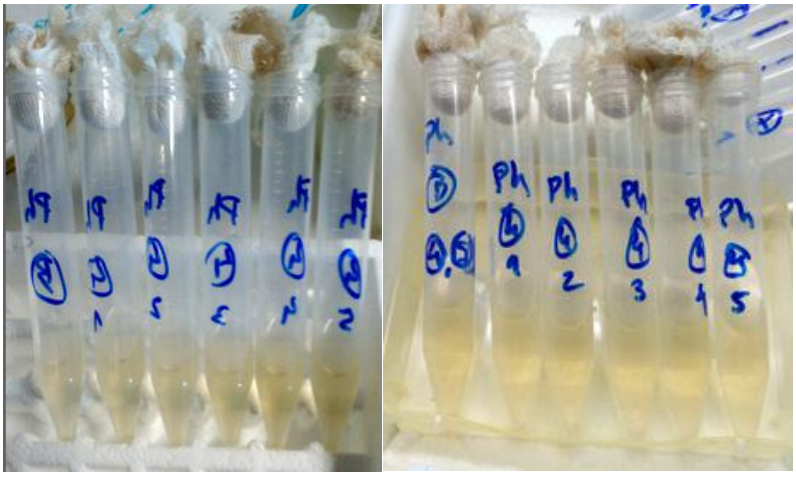


Figure 24 – Samples and control tubes of the first test (left) and second test (right) of the Ph microalga culture with pTSA on the last day of the test

3.2 Benzothiazoles toxicity

3.2.1 Benzothiazole

3.2.1.1 *Dunaliella tertiolecta*

The D microalga started to demonstrate a perceptible green colour inside of the tubes close to the 25th day of culture and macroscopically we could see the same intensity of green colour in every tube, which didn't vary for the subsequent days of culture. The absorbance values measured on the 25th, 34th and 40th days of culture demonstrated that the presence of BT in concentrations equal to or lower than 100ppm didn't affect the growth of the D microalga since in all the measurements the absorbances were similar to each other and to the control (Figure 25).

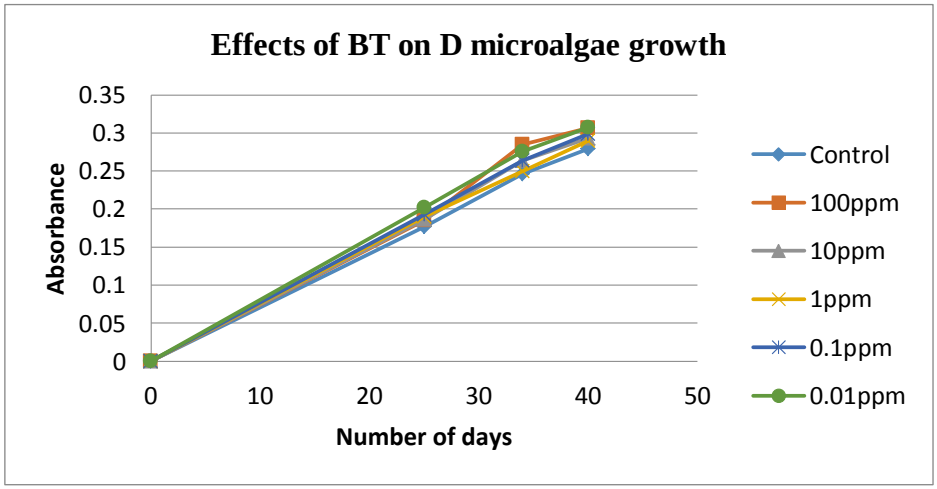


Figure 25 – First test of microalga D growth rates with different concentrations of BT in the medium

On the second test, the results were different. The first absorbance measurement was done on the 20th day and there was a significantly difference in the absorbance values between the cultures containing 100ppm, 50ppm and 25ppm of BT and the rest of the cultures, including the control.

On the 25th day we observed that the tubes were clearly different to each other: the tubes with 100ppm, 50ppm and 25ppm concentrations on BT in the medium had a very slight green colour and the other tubes had a much more perceptible green colour and as we can see by the curves on the graph the D microalga growth was very similar for the three cultures containing the highest concentration of BT, and their growth rate was much lower than the rest of the cultures, including the control. However, on the 32nd day of culture the absorbance values were practically the same for every sample except for the ones containing 100ppm and 50ppm of BT (in which the 50ppm had a lower value than the 100ppm), that were much lower than the rest (Figure 26).

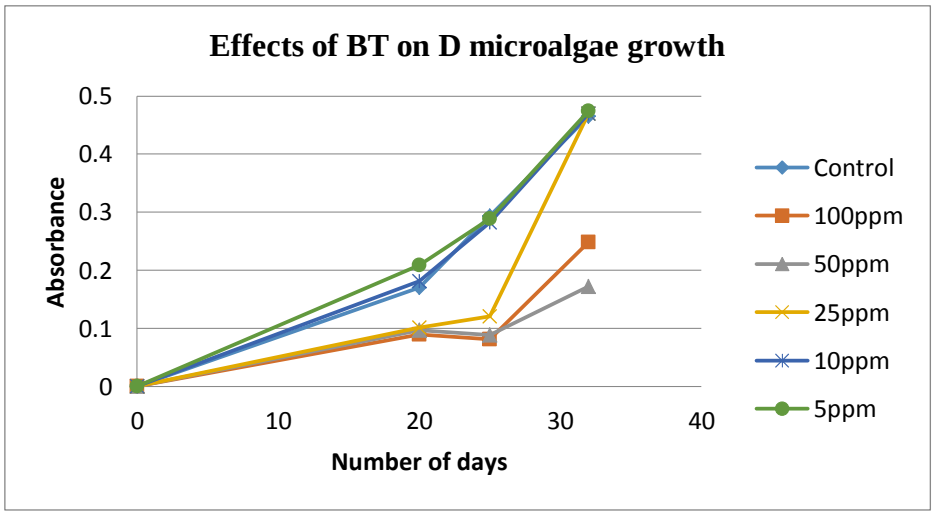


Figure 26 – Second test of microalga D growth rates with different concentrations of BT in the medium

On the first test the D microalga growth wasn't affected by any concentration however, on the second test, 100ppm and 50ppm of BT in the medium (and for unknown reasons 25ppm only until the 25th day of culture) significantly reduced the growth of the microalga. Due to the inconsistency of the results obtained on both tests, further tests should be performed in order to take any conclusions concerning the influence of BT on the D microalga growth.

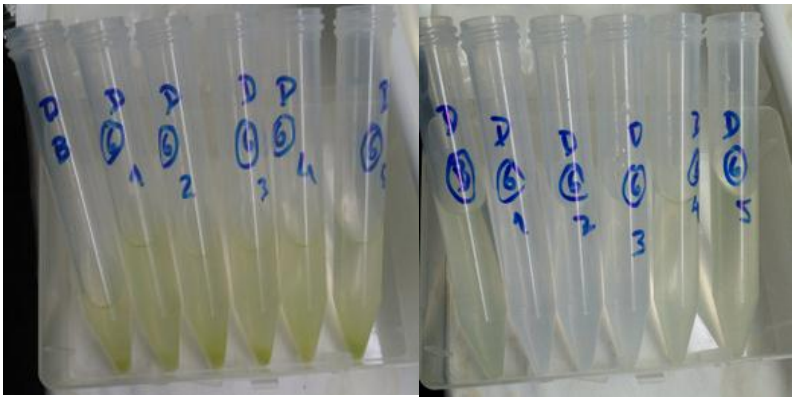


Figure 27 – Samples and control tubes of the first test (left) and second test (right) of the D microalga culture with BT on the 34th and 25th days, respectively

3.2.1.2 *Pseudokirchneriella subcapitata*

On the first test, after reading the absorbances of the samples on the spectrophotometer the results allowed us to take almost the same conclusions as we took after just observing the tubes macroscopically. Only on the 21st and 26th days the absorbance values were significantly different between the samples and the absorbances weren't proportional to the concentration of the pollutant in the medium. A concentration of 100ppm of BT seemed to significantly inhibit the growth of the microalga, but not a concentration of 10ppm that had very similar absorbance values to the control on both days. The lowest concentration of BT (0.01ppm) affected the growth of the K microalga even more than the highest concentration (100ppm). Both concentrations of 0.1ppm and 1 ppm also reduced the growth of the microalga, but this reduction was lower than the one caused by a concentration of 0.01ppm of BT in the medium (Figure 28).

These results didn't allow us to take any conclusions since the influence on the growth of the microalga wasn't proportional, in any way, to the concentrations of the BT in the medium.

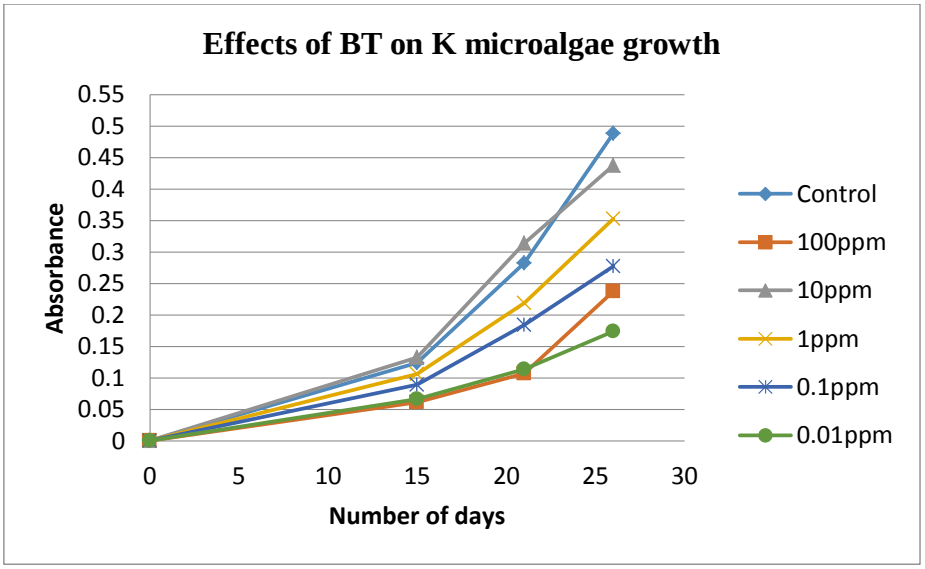


Figure 28 – First test of microalga K growth rates with different concentrations of BT in the medium

For the duration of the whole second test the tubes seemed macroscopically the same and the absorbance values on all three days (21st, 29th and 34th days) were all very similar for all the samples, including the control which indicated that on the second test the BT didn't affect the growth of the K microalga, in the range of concentrations tested (Figure 29).

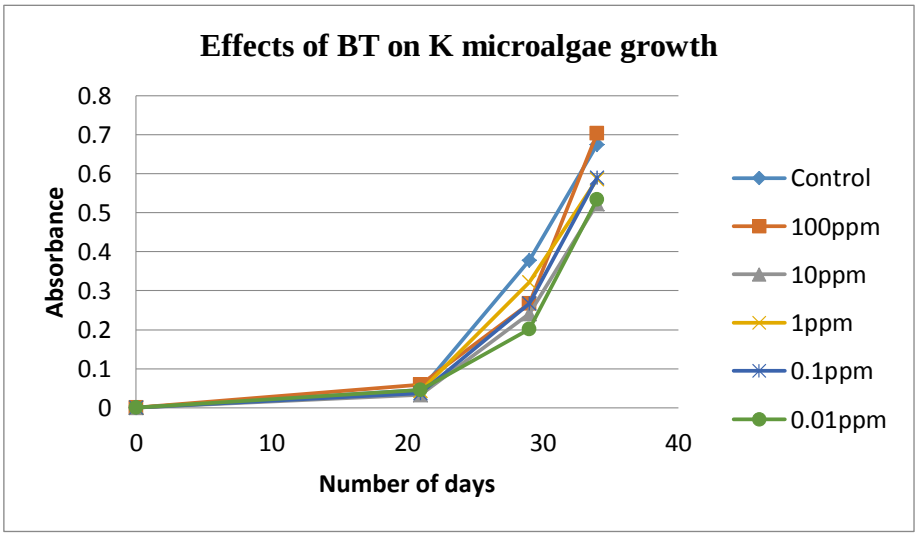


Figure 29 – Second test of microalga K growth rates with different concentrations of BT in the medium

Since that the results on the first test didn't make sense and the results on both tests were completely different we can't take any logical conclusion so further studies should be performed.

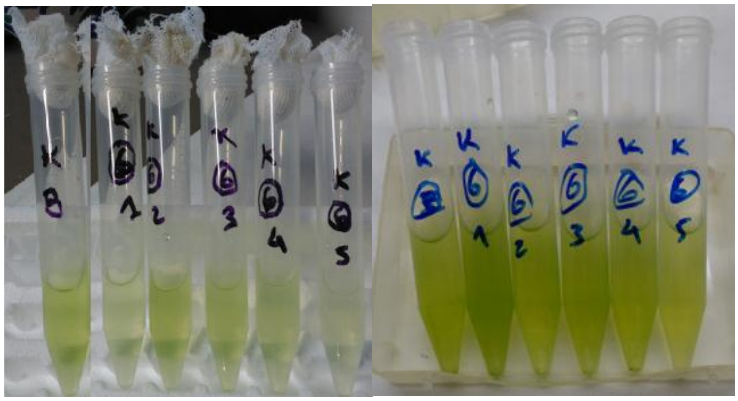


Figure 30 – Samples and control tubes of the first test (left) and second test (right) of the K microalga culture with BT on the last day of the test

3.2.1.3 *Phaeodactylum tricornutum*

On the 15th day of culture, to evaluate the effects of BT on Ph microalgae growth, the tubes already had a very perceptible brown coloration. Macroscopically, the sample containing 100ppm of BT seemed limpid for the duration of the whole test. After reading the absorbances on the 15th, 21st and 26th days we could conclude that in the tube containing a concentration of 100ppm of BT, the Ph microalgae had almost no growth and 10ppm of BT seemed to slightly reduce its growth, while the other samples with lower concentrations of BT had a similar growth rate as the control (Figure 31).

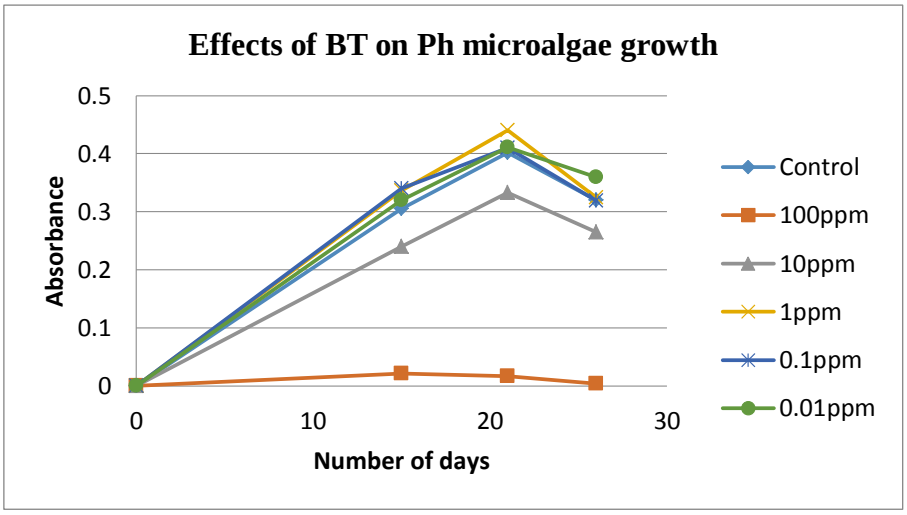


Figure 31 – First test of microalga Ph growth rates with different concentrations of BT in the medium

On the second test the absorbance curves showed that concentrations of BT equal to or higher than 25ppm highly decreased the growth of the Ph microalga, and a 10ppm concentration of this pollutant in the medium moderately affected the growth

of the microalga while a 5ppm concentration had very similar absorbance values to the control, especially on the 29th day of culture, so its effects on the growth of the Ph microalga are not clear (Figure 32).

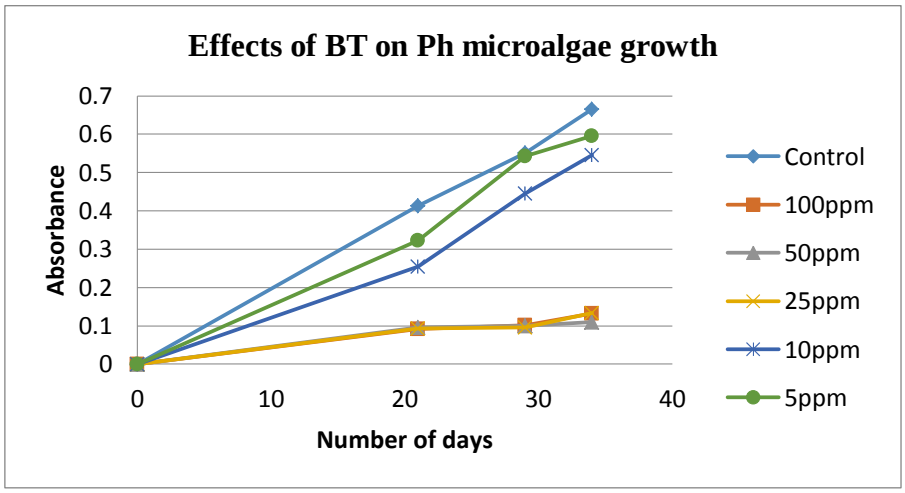


Figure 32 – Second test of microalga Ph growth rates with different concentrations of BT in the medium

In conclusion, by combining the results on both tests, for concentrations equal to or higher than 10ppm the growth of the Ph microalga is affected, which shows that this microalga is sensitive to the BT if its concentration in the medium is equal to or higher than 10ppm. The EC_{50} estimated is between 25 and 10ppm.

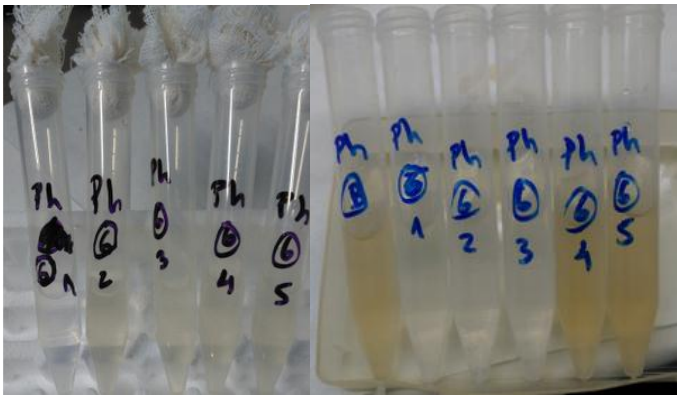


Figure 33 – Samples and control tubes of the first test (left) and second test (right) of the Ph microalga culture with BT on the last day of the test

3.2.2 2-hydroxybenzothiazole

3.2.2.1 *Dunaliella tertiolecta*

On the first test to evaluate the effects of the HOBT on the D microalga growth we could see that for the 39 days of the test all the tubes were very similar to the control except for the one with a concentration of 100ppm of HOBT that a much lighter green colour than the intense green colour of the rest of the tubes. On the 19th, 30th and 39th days of culture we read the absorbances and the results confirmed what we suspected by the macroscopic observation. Only 100ppm of HOBT had a much lower absorbance value than the control and all the other samples absorbances were similar to the control on all the three measurements (Figure 34).

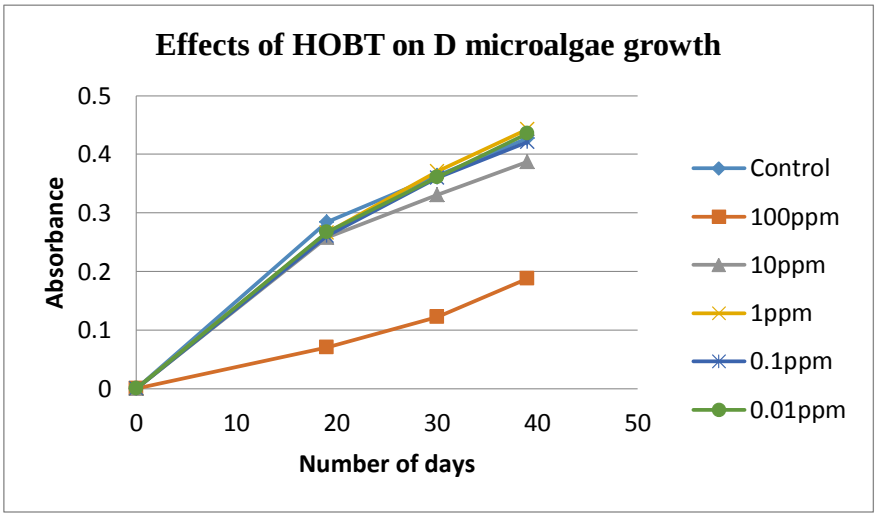


Figure 34 – First test of microalga D growth rates with different concentrations of HOBT in the medium

On the second test, not only the tube with 100ppm of HOBT seemed to have a lighter green colour than the rest of the tubes but also the one containing a 50ppm concentration. After reading the absorbance values, on the 15th, 22nd and 29th days, we can conclude that until the 22nd day the whole range of concentrations tested reduced the growth of the D microalgae, but on the 29th day only the 100ppm concentration had a significant lower absorbance value than the control, so further studies should be performed in order to understand better the effects of 50ppm, 25 ppm, 10ppm and 5ppm concentrations of the HOBT on the growth of the D microalga (Figure 35).

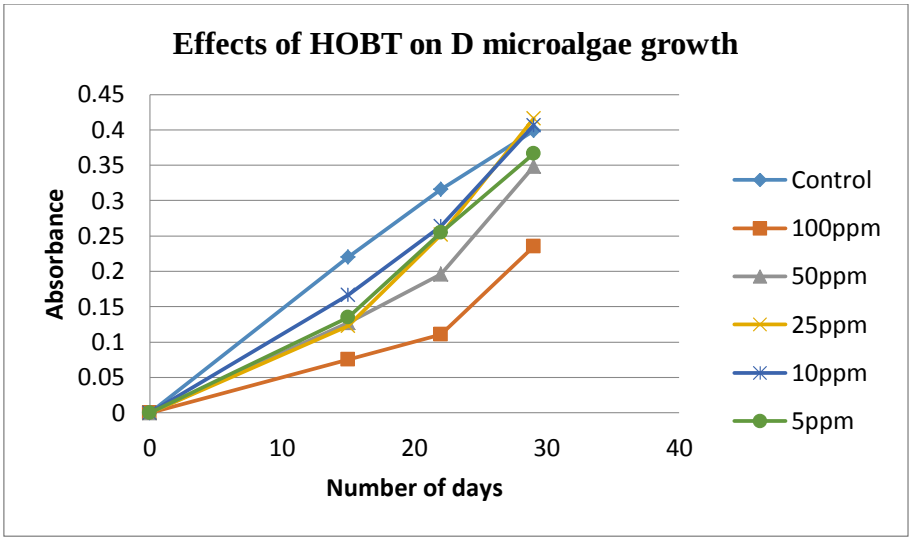


Figure 35 – Second test of microalga D growth rates with different concentrations of HOBT in the medium

With the results of both tests only 100ppm of HOBT clearly decreased the growth of the D microalga so we can only confidently say that the D microalga is sensitive to concentrations of HOBT equal to or higher than 100ppm. The EC_{50} estimated is between 100ppm and 50ppm.

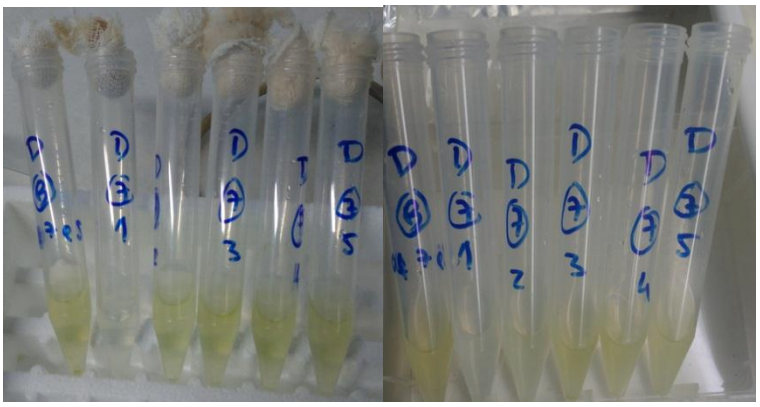


Figure 36 – Samples and control tubes of the first test (left) and second test (right) of the D microalga culture with HOBT on the last day of the test

3.2.2.2 *Pseudokirchneriella subcapitata*

The results on the first measurement of the first test didn't allow us to take any conclusions since the values were low and very similar to each other. On the other hand, on the 21st and 28th days of culture the results were more conclusive. The tubes containing 100ppm and 10ppm had a less intense green coloration, especially on the last day, and the absorbance values were much lower than the control. For

concentrations of 1ppm, 0.1ppm and 0.01ppm the absorbance values were almost equal to the control, and for the concentrations of 10 ppm and 100ppm on the 28th day of culture the growth rate was, approximately, two times and eight times lower than the control, respectively (Figure 37).

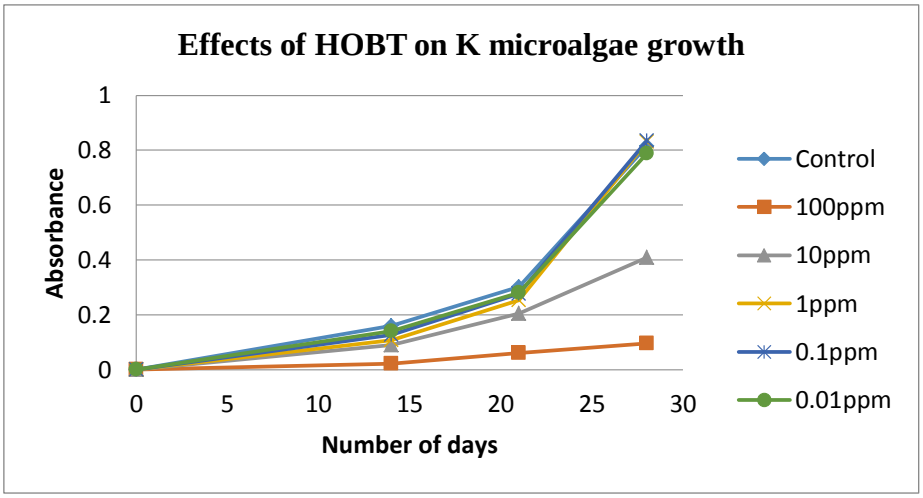


Figure 37 – First test of microalga K growth rates with different concentrations of HOBT in the medium

On the second test the results for the concentrations of 1ppm, 0.1ppm and 0.01ppm were different. On the 22nd and 29th days we could see that not only the growth was affected not only by 100ppm and 10ppm of HOBT in the medium but also by 1ppm, 0.1ppm and 0.01ppm (that caused similar effects as the 10ppm concentration especially on the last day) (Figure 38).

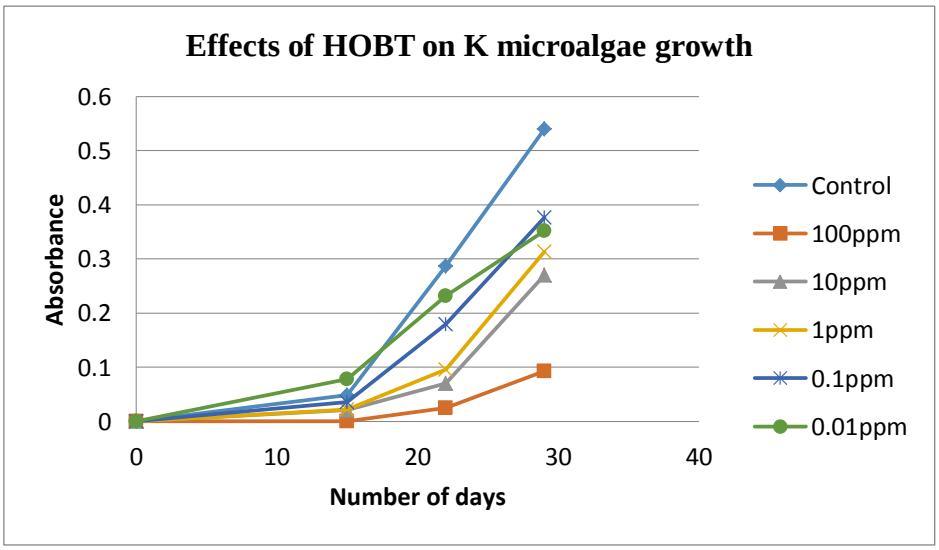


Figure 38 – Second test of microalga K growth rates with different concentrations of HOBT in the medium

Combining the results of both tests, we can conclude that the K microalgae growth is reduced in the presence of concentrations of HOBT in the water higher than or equal to 10ppm, and since the studies weren't conclusive to the concentrations lower than 10ppm further studies should be done to evaluate the influence of these concentrations of HOBT on the growth of this alga. The EC_{50} estimated is approximately 10ppm.

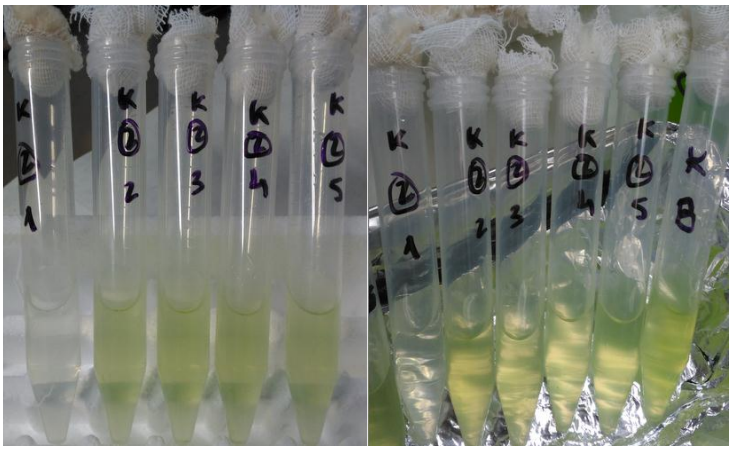


Figure 39 – Samples and control tubes of the first test (left) and second test (right) of the K microalga culture with HOBT on the 21st and 22nd days, respectively

3.2.2.3 *Phaeodactylum tricornutum*

On the first test, the absorbances were analysed on the 14th, 19th and 30th days of culture and in all of them the conclusions were the same: the absorbances of all the samples containing 0.01ppm to 100ppm of HOBT were similar to the control with the exception of the 10ppm sample that had a significantly lower value. These results weren't conclusive about the effects of the HOBT on the Ph microalga since that the only concentration that decreased the growth of this microalga was 10ppm, but 100ppm didn't, which isn't in concordance with our initial premise that the reduction on the growth of the microalga should be proportional to the concentration of the pollutant (Figure 40).

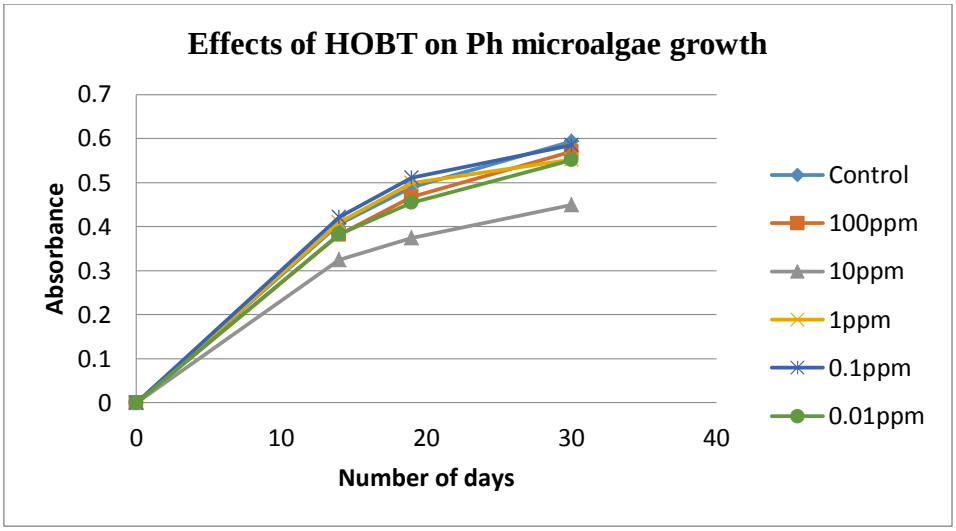


Figure 40 – First test of microalga Ph growth rates with different concentrations of HOBT in the medium

On the second test, the absorbance results showed what we expected with the macroscopic results: on the 15th day the absorbance values for the tubes with concentrations of 10ppm and higher of HOBT were close to zero while the 5ppm tube and the control showed a significant growth of the Ph microalga in the medium (the control with a higher growth rate than the 5ppm sample). On the 21st day of culture the conclusions were similar to the previous ones except for the concentration of 10ppm that now had an absorbance value close to the 5ppm culture. On the 29th only the 100ppm and 50ppm concentrations of HOBT caused almost no growth of the Ph microalga and the reduction on the growth of the rest of the samples was proportional to the concentration of HOBT in the medium (although the 10ppm and 5ppm concentrations had a very similar absorbance value) with the lowest concentration reducing the growth in about 35% in the end of the test, when compared to the control (Figure 41).

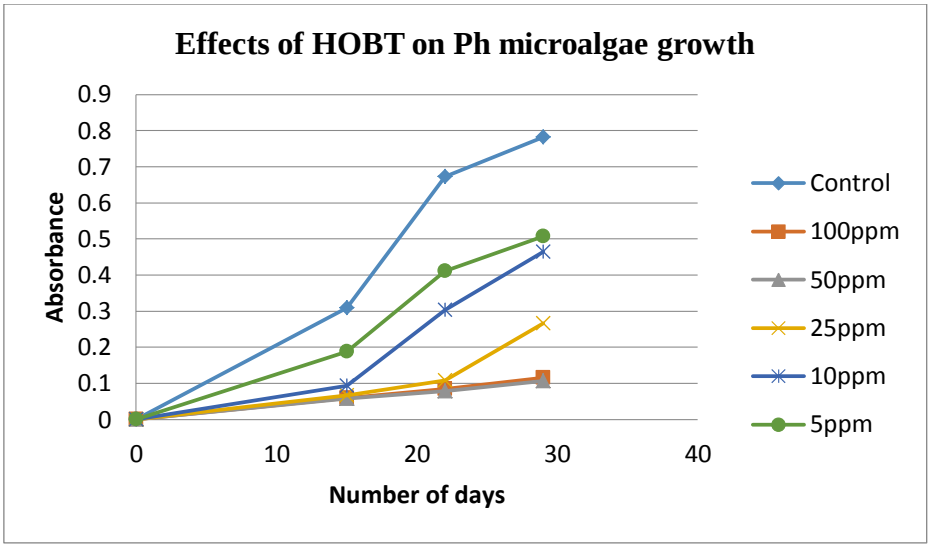


Figure 41 – Second test of microalga Ph growth rates with different concentrations of HOBT in the medium

Although the results on the second test made sense since the reduction on the microalga growth was proportional to the concentration of HOBT in the medium, the lack of concordance of the two tests (mostly for the concentrations of 100ppm and 10ppm that were common on both tests) doesn't allow us to take any conclusion about the effects of the HOBT on the Ph microalga growth and so further studies should be performed to evaluate why the two tests of this study had such different results.

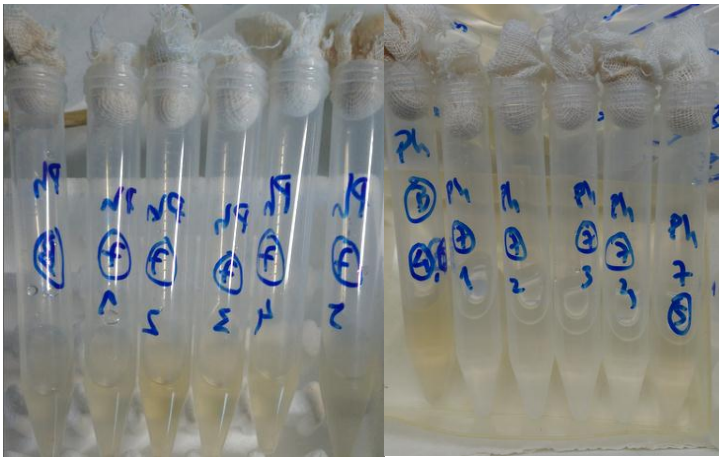


Figure 42 – Samples and control tubes of the first test (left) and second test (right) of the Ph microalga culture with HOBT on the 19th and 22nd days, respectively

3.2.3 2-(methylthio)benzothiazole

3.2.3.1 *Dunaliella tertiolecta*

On all the three days of spectrophotometry analyses, on the first test, the absorbance values of the samples were very close to each other and to the control, apart from the one with 100ppm of MeSBT that had a much lower absorbance value, especially on the 25th day (Figure 43).

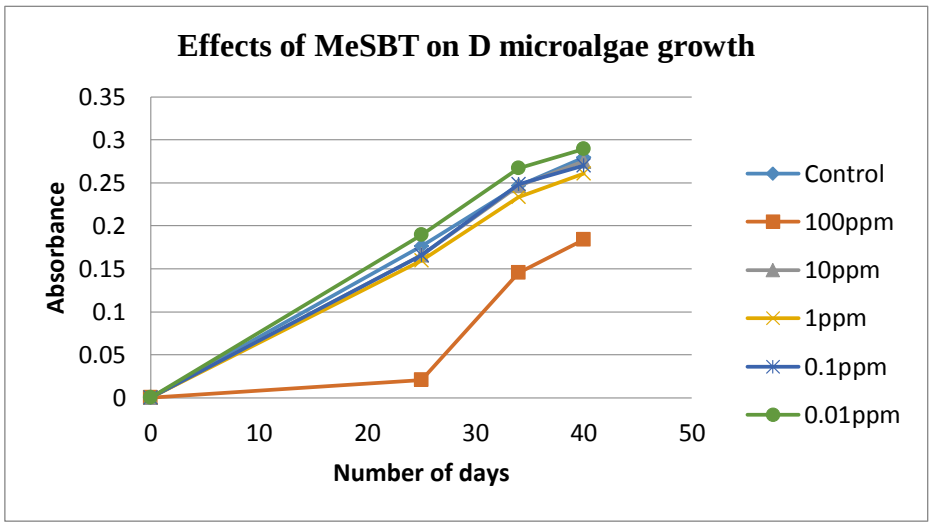


Figure 43 – First test of microalga D growth rates with different concentrations of MeSBT in the medium

On the second test, with a different range of concentrations, the results were similar to the first test except for the fact that not only 100ppm of MeSBT affected the growth of the D microalga but also the 50ppm concentration reduced the growth of this microalga on the same proportion, which could be assumed by the less intense green colour on these two tubes (compared to the other four) and the absorbance values were significantly lower than the control and the other samples with lower concentrations of MeSBT (Figure 44).

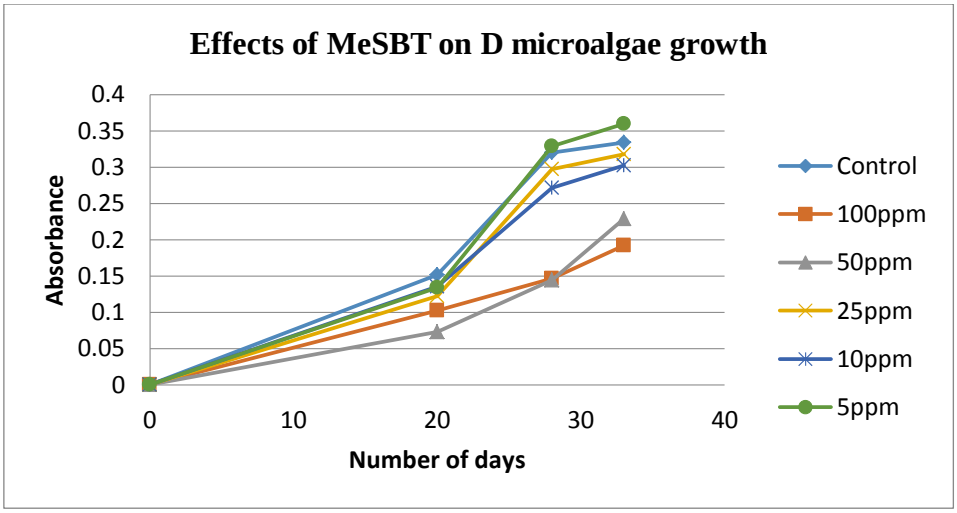


Figure 44 – Second test of microalga D growth rates with different concentrations of MeSBT in the medium

The conclusions taken by these two tests were that the D microalga growth is affected in the presence of MeSBT in the medium. This microalga is sensitive to concentrations higher or equal to 50ppm of MeSBT in the water and the EC_{50} estimated is higher than 100ppm.

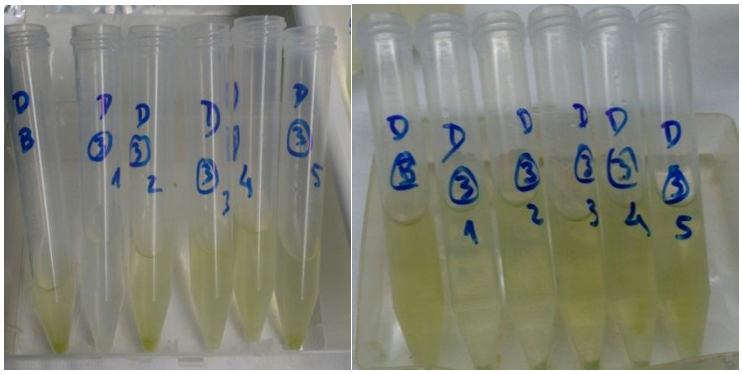


Figure 45 – Samples and control tubes of the first test (left) and second test (right) of the D microalga culture with MeSBT on the last day of the test

3.2.3.2 *Pseudokirchneriella subcapitata*

On the first test, the absorbance results on the 15th day of culture weren't conclusive due to the fact that all the absorbance values were low and similar to each other. On the 21st day the concentration of 100ppm of MeSBT caused a significantly lower growth of the microalga when compared to the rest of the samples and concentrations of 1ppm, 0.1ppm and 0.01ppm had very similar absorbance values, which were significantly lower than the control (and although it would be expected that 10ppm of MeSBT to also inhibit the growth of the K microalga and on a higher

level than lower concentrations it had a higher absorbance value than the control itself). On the 26th day we still registered the lowest absorbance for the 100ppm sample, which continued to be significantly lower than the rest of the samples and although the absorbance value of the 10ppm sample was this time significantly lower than the control, it was still higher than the absorbances registered for lower concentrations than 10ppm of MeSBT (Figure 46).

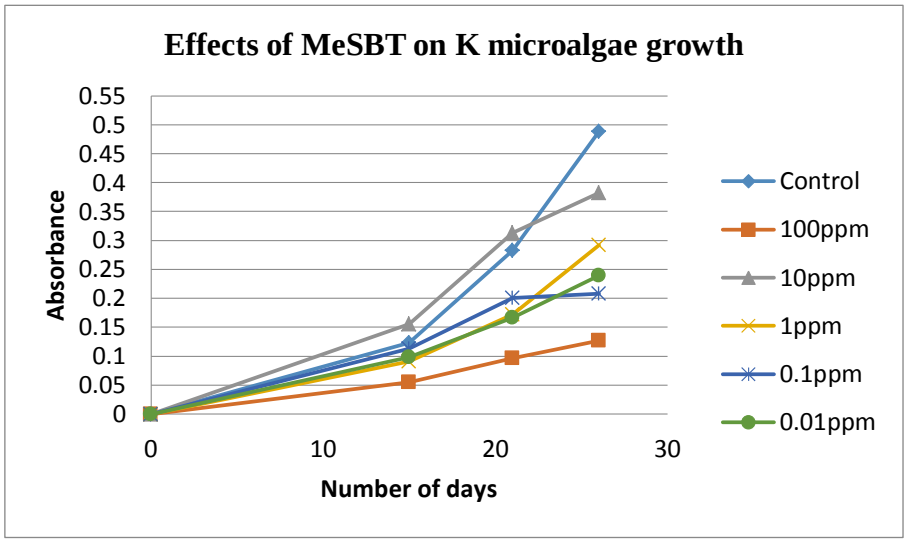


Figure 46 – First test of microalga K growth rates with different concentrations of MeSBT in the medium

On the second test, with the same range of concentrations, the results were different than the ones on the first test. Although we didn't have a perceptible green colour in the tubes on the 21st day of culture we proceeded with the firsts spectrophotometric readings so that the absorbance measurement days of the second test wouldn't be so different than the ones on the first test. With the spectrophotometric results on the 29th and 34th we could conclude that all the absorbances were lower than the control but the reduction on the growth of the microalga wasn't proportional to the concentration of MeSBT in the medium. On the last measurement the 100ppm concentration caused a significantly lower growth of the microalga than the rest of the concentrations and a concentration of 1ppm also had a significantly lower absorbance value than the control (the 10ppm, 0.1ppm and 0.01ppm also affected the growth of the microalga although not as much as 100ppm and 1ppm of MeSBT) (Figure 47).

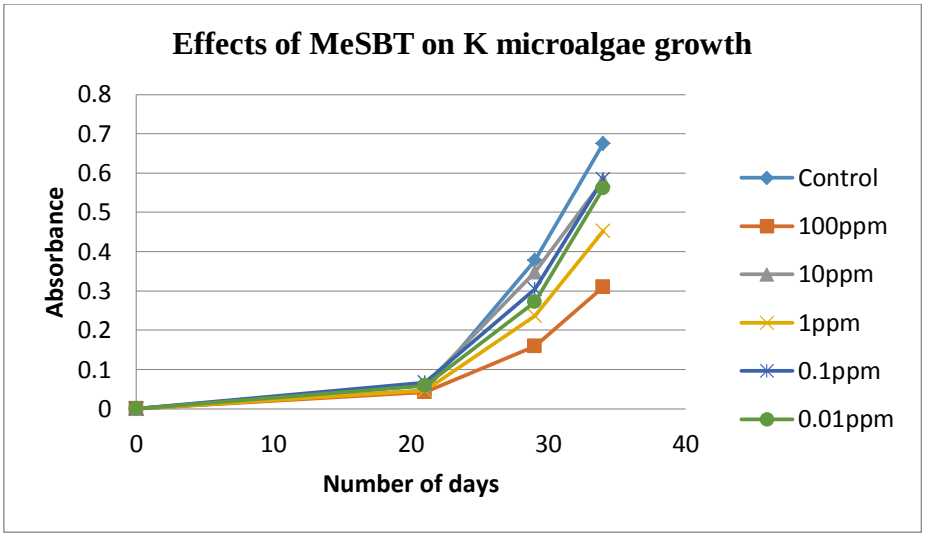


Figure 47 – Second test of microalga K growth rates with different concentrations of MeSBT in the medium

Combining the results of both tests, only the concentration of 100ppm of MeSBT affected the growth of the microalga in similar ways on both tests, while the other concentrations showed different results and the reduction wasn't proportional to the concentration of MeSBT in the medium, so we can only assume that the K microalga is sensitive to concentrations of 100ppm or higher of MeSBT and for lower concentrations further studies should be performed. The EC_{50} estimated is lower than 100ppm (since the results were not conclusive for concentrations lower than 100ppm we can't calculate a more precise value).

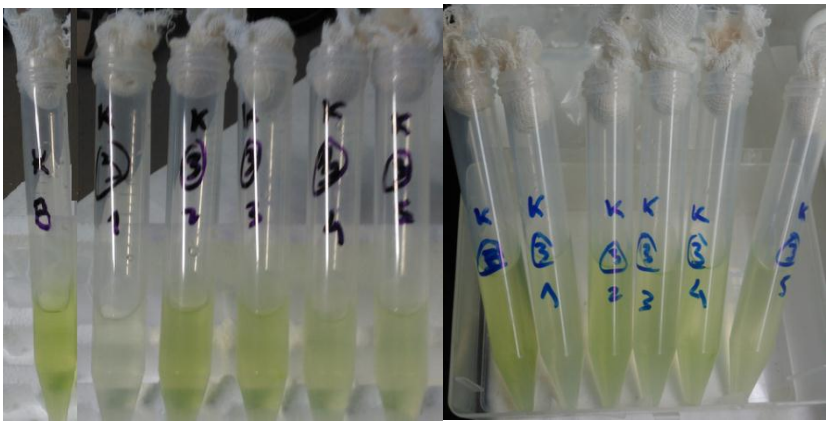


Figure 48 – Samples and control tubes of the first test (left) and second test (right) of the K microalga culture with MeSBT on the last day of the test

3.2.3.3 *Phaeodactylum tricornutum*

On the 15th day of the first test we proceeded with the first absorbance measurement and the conclusions were the same for all the three measurements (on the 15th, 21st and 26th days): the absorbances were similar for the control and all the samples except for the concentration of 100ppm that registered a significantly lower absorbance value than the control in all the three days (especially on the first two since that as explained before, after the second measurement, 1mL of purified water was added to all the tubes to avoid the concentration of the medium that could affect the growth of the microalga, which led to a dilution of the medium) (Figure 49).

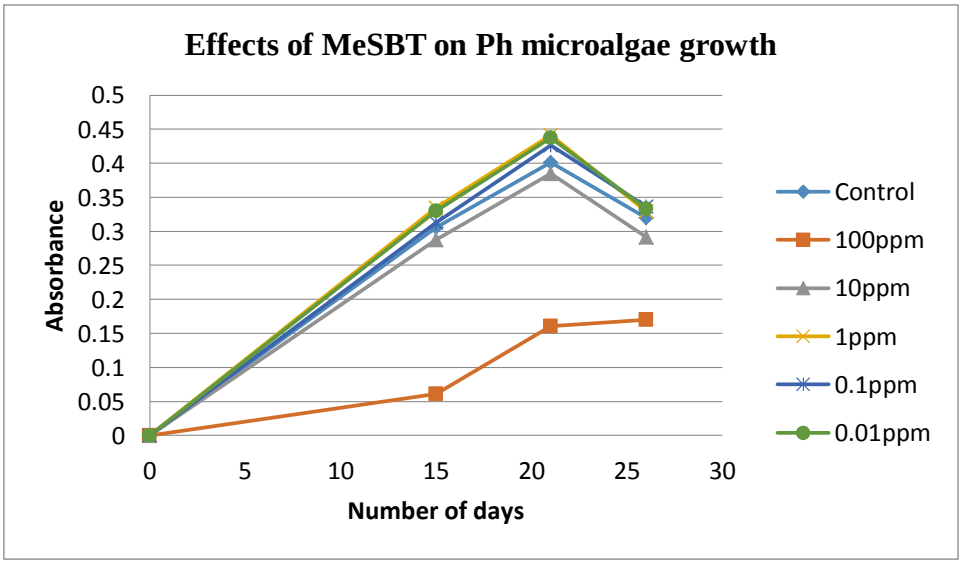


Figure 49 – First test of microalga Ph growth rates with different concentrations of MeSBT in the medium

On the second test, all the concentrations reduced the growth of the Ph microalga and on a proportional level. In all three measurements (on the 21st, 29th and 34th days) 100ppm, 50ppm and 25ppm of MeSBT in the medium highly affected the growth of the microalga and the reduction was proportional to the concentration of the pollutant in the medium. On the other hand, 10ppm and 5ppm of MeSBT also reduced the growth of the microalga but it was similar for both (and not a higher reduction for 10ppm as expected) and much lower than the other three concentrations, for example, on the second measurement it was lower than 15% for both 10ppm and 5ppm (Figure 50).

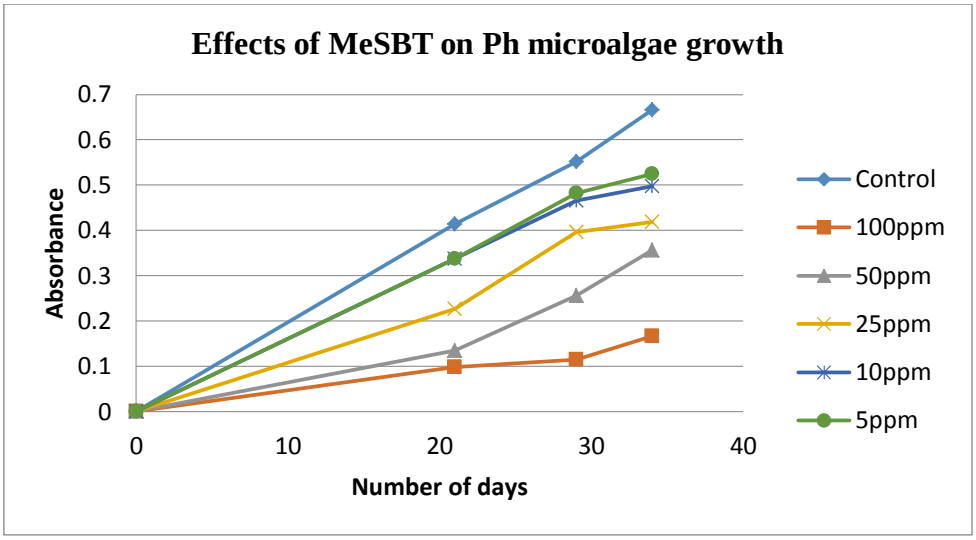


Figure 50 – Second test of microalga Ph growth rates with different concentrations of MeSBT in the medium

With the results on both tests we can say that the Ph microalga is sensitive to concentrations of 25ppm and higher of MeSBT in the water (we can't say with no doubts that the Ph microalga is also sensitive to concentrations of 10ppm and 5ppm since that the reduction on the second test was much lower than for the rest of the concentrations, and on the first test the 10ppm concentration didn't affect the growth of the microalga). The EC_{50} estimated of the MeSBT for the Ph microalga is between 100ppm and 50ppm.

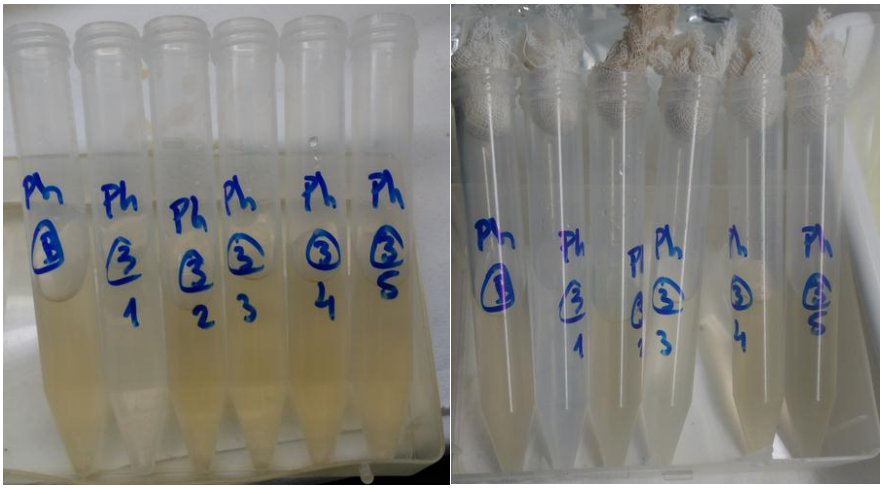


Figure 51 – Samples and control tubes of the first test (left) and second test (right) of the Ph microalga culture with MeSBT on the 21st and 29th days, respectively

3.3 Benzotriazoles toxicity

3.3.1 Benzotriazole

3.3.1.1 *Dunaliella tertiolecta*

On the first test, the tubes started to show a green perceptible coloration near the 25th day of culture. We could see a clear difference between the tube with a concentration of 100ppm of BTR and the five other tubes that showed a more intense colour. On the 34th and 40th days this difference was lower.

The results obtained by spectrophotometry allowed us to take the same conclusions as the ones suspected macroscopically. For concentrations of BTR lower than 100ppm the growth of the Ph microalga was not affected, but for a concentration of 100ppm the growth was significantly lower than the control (Figure 52).

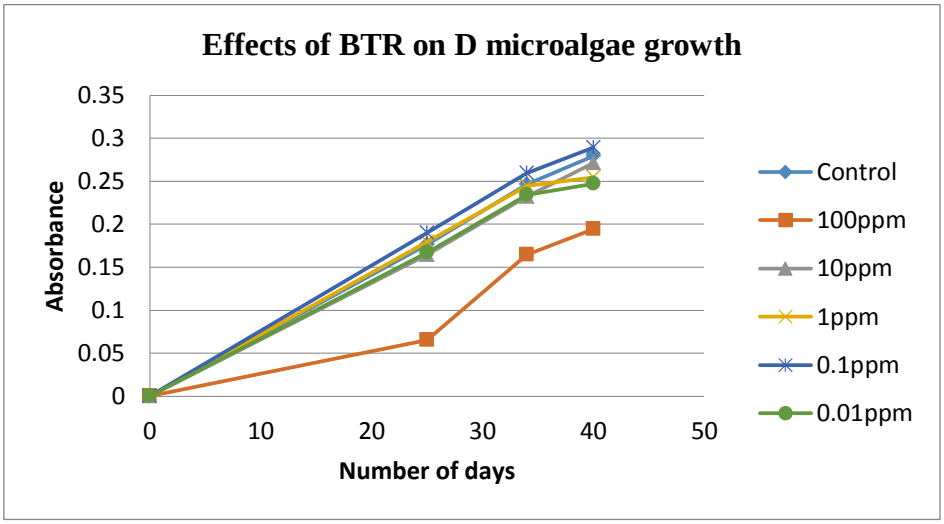


Figure 52 – First test of microalga D growth rates with different concentrations of BTR in the medium

On the second test, the first absorbance measurement was done on the 20th day and the other two measurements on the 25th and 33rd days. The absorbance values allowed us to conclude that not only the growth rate was lower with 100ppm of BTR in the medium but also with 50ppm especially on the first two measurements since that on the third one the absorbance value of the 50ppm sample was closer to the control, but still lower (Figure 53).

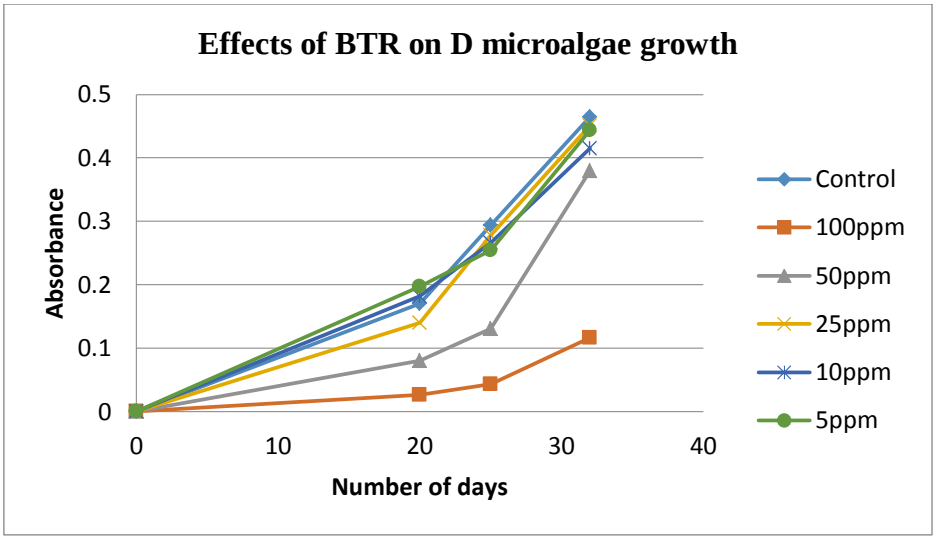


Figure 53– Second test of microalga D growth rates with different concentrations of BTR in the medium

With both tests we can affirm that the presence of BTR in the medium affects the growth of the Ph microalga when the concentrations of this pollutant are higher or equal to 50ppm. The EC_{50} of the BTR for the Ph microalga was estimated to be between 100ppm and 50ppm.

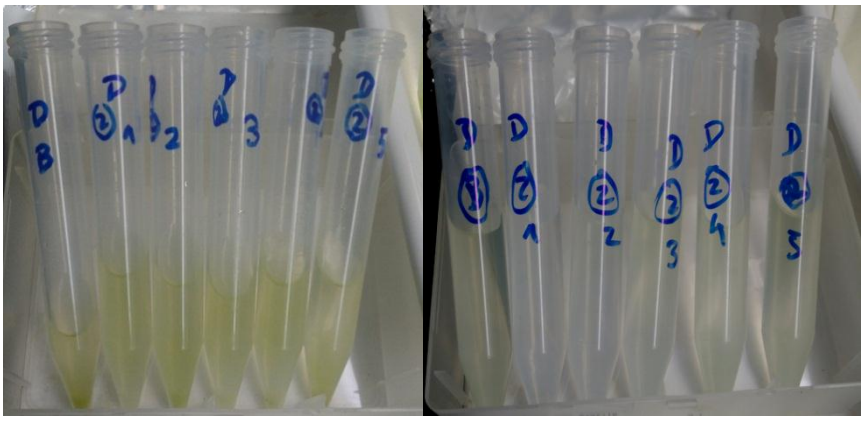


Figure 54 – Samples and control tubes of the first test (left) and second test (right) of the D microalga culture with BTR on the 34th and 25th days, respectively

3.3.1.2 *Pseudokirchneriella subcapitata*

On the 15th of this first test we measured the first absorbance values and they were all similar to each other and the control.

On the 21st day we could already see differences between the tubes and the control, both macroscopically and by comparing the absorbance values. For unknown reasons, the absorbance of the 100ppm sample on the second measurement was lower

than on the first one, maybe due to an error on the first measurement since that on the third measurement the absorbance value is still lower. On this second measurement, as well as on the third one, all the absorbance values were lower than the control and similar to each other except for the 100ppm sample that had a value close to zero while the other samples registered values between 0.15 and 0.2 on the 21st day and between 0.25 and 0.3 on the 26th day (Figure 55).

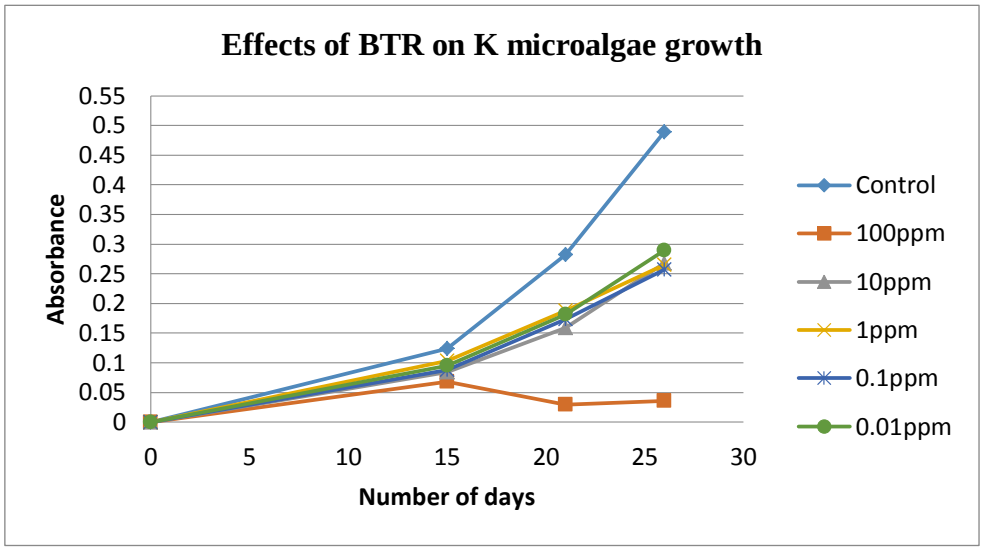


Figure 55 – First test of microalga K growth rates with different concentrations of BTR in the medium

On the 21st day of the second test we proceeded with the first spectrophotometric measurements but the absorbance values were very low and similar to each other (macroscopically the tubes looked limpid but in order to don't get the days of the measurements too apart from the ones on the first test we decided to initiate the absorbance measurements). The absorbance values on the second measurement, on the 29th day of culture, as well as on the last measurement, were different than on the first test since that this time for concentrations lower than 100ppm (100ppm of BTR continued to highly inhibit the growth of the K microalga) the growth of the microalga was only slightly reduced and the reduction wasn't even proportional to the concentration of BTR in the medium since that the 10ppm sample had an absorbance value similar to the control, while the 1ppm, 0.1ppm and 0.01ppm samples had a lower absorbance value than the 10ppm sample (Figure 56).

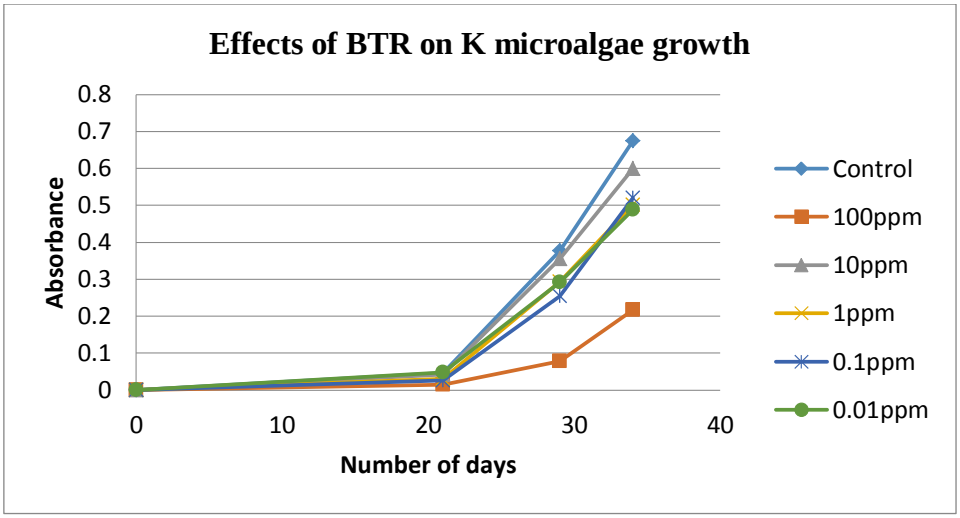


Figure 56 – Second test of microalga K growth rates with different concentrations of BTR in the medium

With the results of both tests we can conclude that the K microalga is sensitive to concentrations of BTR of 100ppm or higher and for lower concentrations than 100ppm more studies should be performed since that the results on this study weren't conclusive about the effects of concentrations between 10ppm and 0.01ppm of BTR on the growth of the K microalga. The EC_{50} estimated for the BTR on the K microalga is lower than 100ppm and since that we don't have conclusive results for lower concentrations, a more exact result should be investigated on further studies.

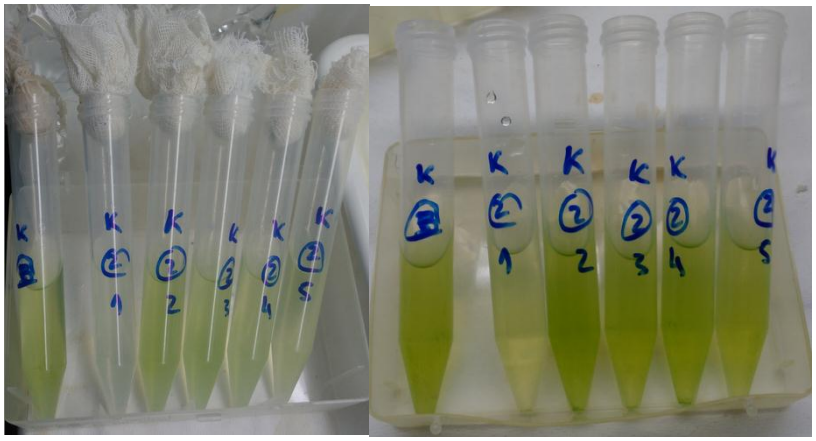


Figure 57 – Samples and control tubes of the first test (left) and second test (right) of the K microalga culture with BTR on the last day of the test

3.3.1.3 *Phaeodactylum tricornutum*

After analysing the absorbance results of the first test, the conclusions were the same for all the three measurements (on the 15th, 21st and 26th days): the absorbances were similar for the control and all the samples, except for the concentration of 100ppm that had a much lower absorbance value in all the three days (the lower absorbances on the third measurement for all the samples except for the 100ppm tube were due to the dilution of the medium with 1mL of purified water as explained before) (Figure 58).

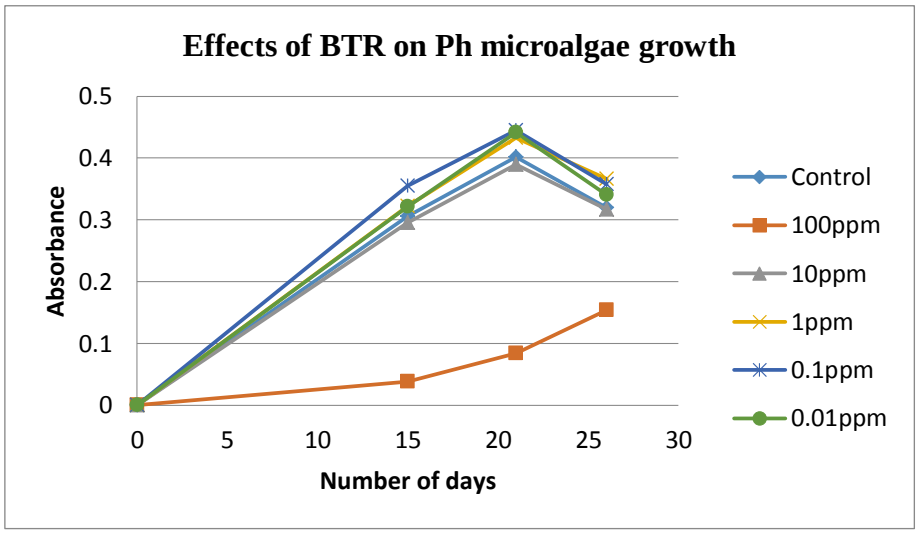


Figure 58 – First test of microalga Ph growth rates with different concentrations of BTR in the medium

On the second test, with a different range of concentrations, all of them seemed to inhibit the growth of the Ph microalga. In all three measurements (on the 21st, 29th and 34th days) 100ppm, 50ppm and 25ppm of BTR in the medium highly affected the growth of the microalga and the reduction was proportional to the concentration of the pollutant in the medium. On the other hand, 10ppm and 5ppm of BTR also reduced the growth of the microalga (but it wasn't higher for 10ppm as expected) however the reduction was significantly lower than the other three concentrations, for example, on the second measurement the reduction was lower than 10% for the 10ppm concentration and almost 0% for the 5ppm concentration (Figure 59).

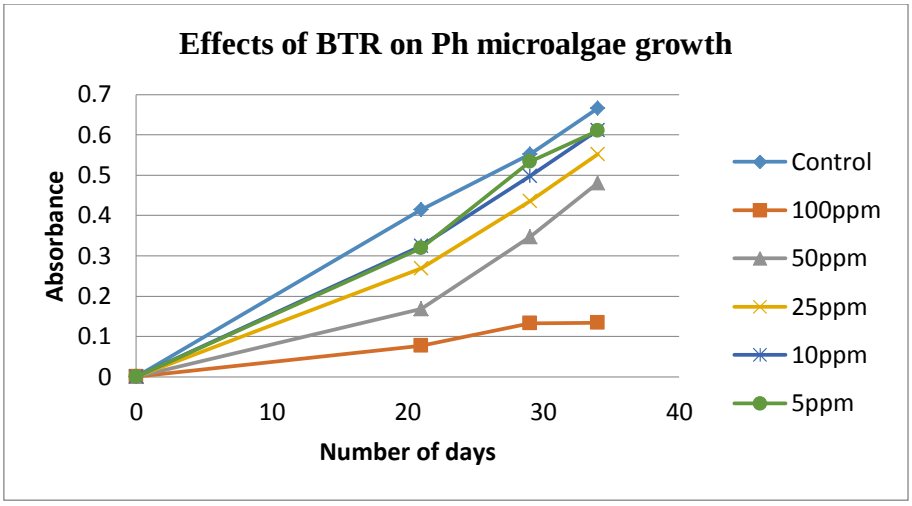


Figure 59 – Second test of microalga Ph growth rates with different concentrations of BTR in the medium

With the results on both tests we can say that the Ph microalga is sensitive to concentrations of 25ppm and higher of BTR in the medium. The EC_{50} estimated of the BTR for the Ph microalga is between 100ppm and 50ppm.

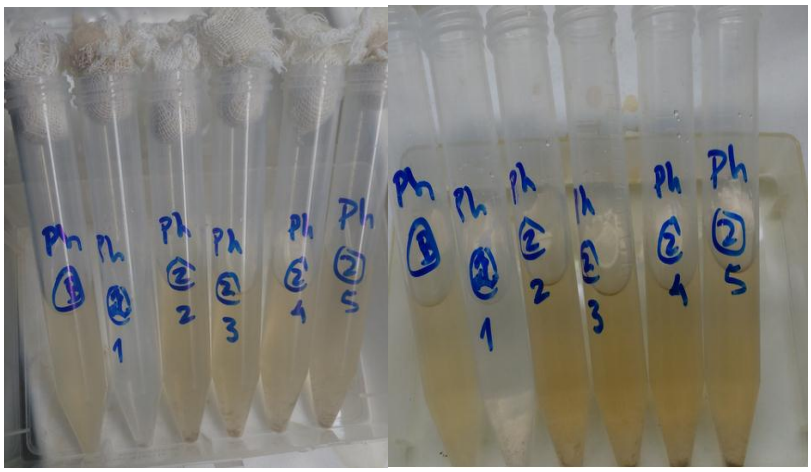


Figure 60 – Samples and control tubes of the first test (left) and second test (right) of the Ph microalga culture with BTR on the last day of the test

3.3.2 5-methylbenzotriazole

3.3.2.1 *Dunaliella tertiolecta*

On the 19th day we proceeded with the first absorbance measurements and the results were similar for all the three days of spectrophotometric measures (19th, 30th and 39th days of culture) since in all three of them the absorbance values of every sample were similar to each other and to the control with the exception of the concentration of 100ppm that significantly decreased (although the reduction was

only of approximately 20% on the 39th day) the growth of the D microalga when compared to the absorbance values of the control. Macroscopically, there were no perceptible differences between the tubes (Figure 61).

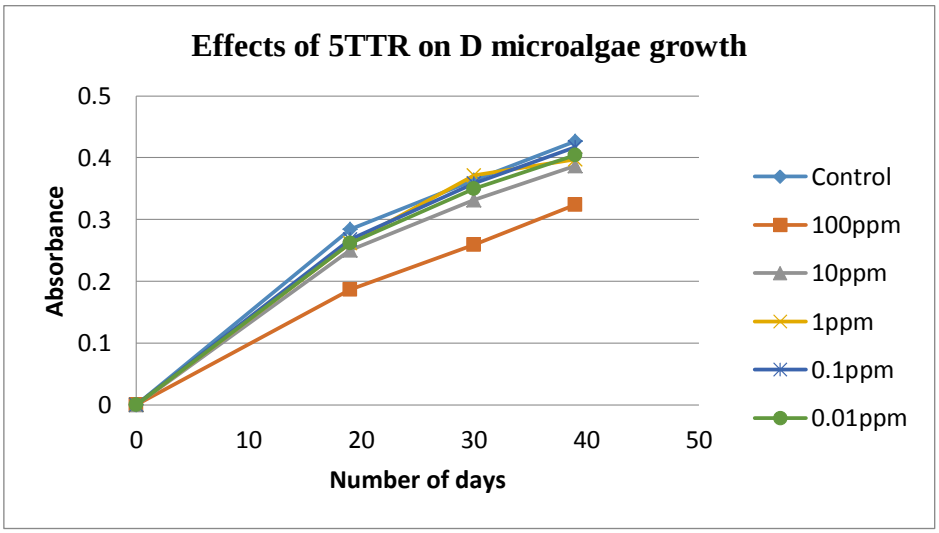


Figure 61 – First test of microalga D growth rates with different concentrations of 5TTR in the medium

Even with a different range of concentrations, on the second test, macroscopically there wasn't a perceptible difference between the tubes with 5TTR and the control tube. With the results of the spectrophotometric analysis on the 15th and 22nd days we could see that the absorbance values of the samples containing 5TTR in the medium were lower than the control, and the reduction on the growth of the microalga was proportional to the concentration of 5TTR in the medium (with the exception of the sample with 10ppm on the 22nd day that had a lower absorbance value than the sample with a higher concentration of 5TTR, 25ppm). However, on the 29th day the absorbance values of the samples weren't significantly lower than the control (that would allow us to say with no doubts that they cause a significant reduction on the growth of the D microalga) with the exception of the 100ppm of 5TTR sample, which caused a reduction on the growth in about 20% on the last absorbance measurement, like on the first test, when compared to the control (Figure 62).

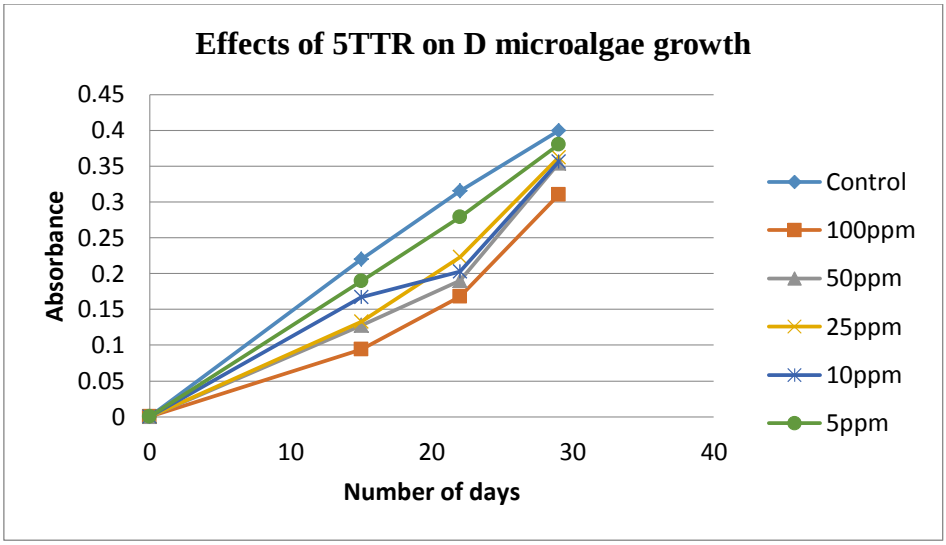


Figure 62 – Second test of microalga D growth rates with different concentrations of 5TTR in the medium

Combining the results of the two test we can conclude that concentrations of 100ppm or higher of 5TTR in the water affects the growth of the D microalga. The EC_{50} estimated is higher than 100ppm of 5TTR.

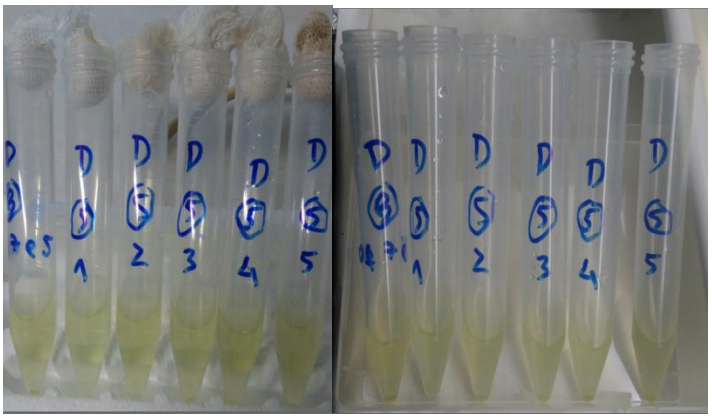


Figure 63 – Samples and control tubes of the first test (left) and second test (right) of the D microalga culture with 5TTR on the last day of the test

3.3.2.2 *Pseudokirchneriella subcapitata*

On the first test, on the 14th and 21st days of culture, the absorbance value of the 100ppm sample was close to zero and the rest of the samples had absorbance values between similar to each other and the control.. The absorbance values on the last day demonstrated that the presence of 100ppm of 5TTR in the medium reduced the growth of the K microalga in almost 90%, and for lower concentrations (that didn't affect its growth until the end of the test) absorbance values started to differ from each other and the control. For example, on the last measurement the

concentration of 10ppm reduced the growth of the microalga in about 30% and the 0.01ppm in about 10%, however due to the fact that only on the last measurement lower concentrations than 100ppm of 5TTR seemed to affect the growth of the microalga (and in a much lower percentage than the 100ppm concentration did) we can't guarantee that the K microalga is actually sensitive to lower concentrations of 5TTR than 100ppm (Figure 64).

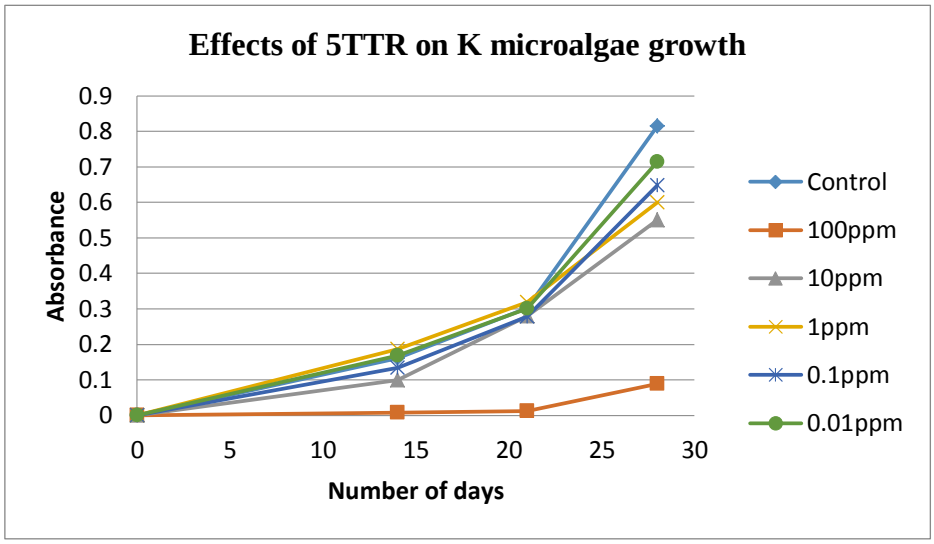


Figure 64 – First test of microalga K growth rates with different concentrations of 5TTR in the medium

On the second test, only the conclusions for the 100ppm concentrations were the same than on the first test: the absorbance values were much lower than the control which indicates that a concentration of 100ppm of 5TTR in the medium highly reduces the growth of the K microalga, like we concluded on the first test. For concentrations lower than 100ppm the results were confusing since the reduction on the growth wasn't proportional to the concentration of 5TTR in the medium: on the second measurement the 10ppm sample had an absorbance value similar to the 100ppm sample (which didn't happen on the first test) while the rest of the samples had a higher value and close to the control. On the third measurement we could see that the 1ppm sample had a higher absorbance value than the control while lower concentrations (0.1ppm and 0.01ppm) registered significantly lower absorbances than the control (Figure 65).

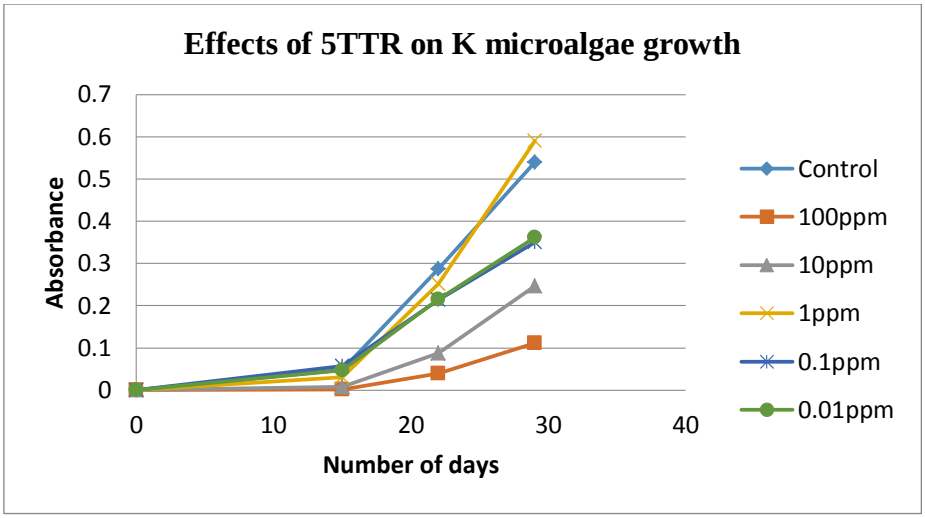


Figure 65 – Second test of microalga K growth rates with different concentrations of 5TTR in the medium

With the inconsistency of the results on both tests for concentrations lower than 100ppm we can only conclude that the K microalgae are sensitive to concentrations of 100ppm or higher of 5TTR in the water, and to lower concentrations than 100ppm more studies should be performed in order to obtain conclusive results. The EC_{50} estimated is lower than 100ppm and a more exact value should be investigated on further studies since that the results for lower concentrations than 100ppm were inconclusive in this study.

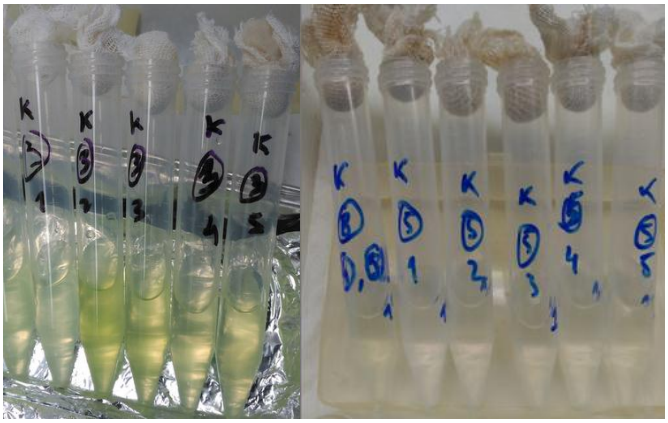


Figure 66 – Samples and control tubes of the first test (left) and second test (right) of the K microalga culture with 5TTR on the 14th and 15th days, respectively

3.3.2.3 *Phaeodactylum tricornutum*

The absorbance results on the 14th day showed the same results as the macroscopic observations with all the samples having similar absorbance values than the control (although the samples with 10ppm and 0.01ppm had a lower absorbance than the control it wasn't as significant as the 100ppm sample) while the sample with 100ppm of 5TTR in the medium had a value close to zero (Figure 67).

On the 19th and 30th days of culture the results were similar to each other but different than on the 14th since that the absorbance values started to differ: while the absorbance value of the 100ppm sample remained close to zero (demonstrating almost no growth of the Ph microalga in the medium) the 10ppm concentration had absorbance values of approximately half of the values of the control. Concentrations lower than 10ppm also affected the growth of the microalga however the reduction on its growth wasn't proportional to the concentration of 5TTR in the medium since that the 0.01ppm sample had a lower absorbance values than the 0.1ppm and both were also lower than the 1ppm absorbance values (Figure 67).

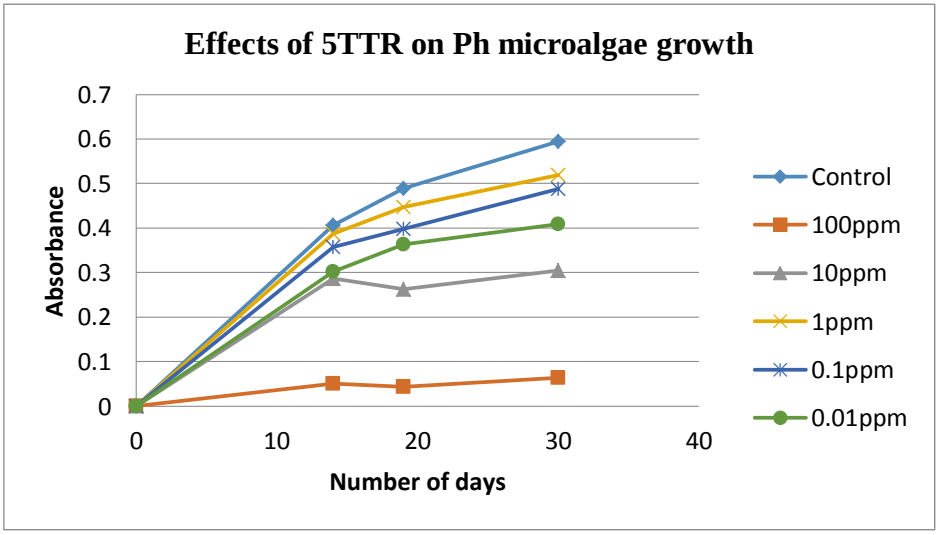


Figure 67 – First test of microalga Ph growth rates with different concentrations of 5TTR in the medium

We repeated the test, now with a range of concentrations from 100ppm to 5ppm and the absorbance results the 15th, 22nd and 29th days allowed us to conclude that all the concentrations of 5TTR on this test caused a reduction on the growth of the Ph microalga in the medium, and although we can't say that the absorbance values were completely directly proportional to the concentration of 5TTR in the medium (since that the reduction on the growth caused by the 100ppm concentration was

similar to the 50ppm concentration and, especially on the 29th day, the 10ppm and 5ppm samples also had similar values), higher concentrations of 5TTR led to lower absorbance values with no exceptions (there was no sample with a higher concentration having a higher absorbance value than a sample with a lower concentration of 5TTR in the medium) (Figure 68).

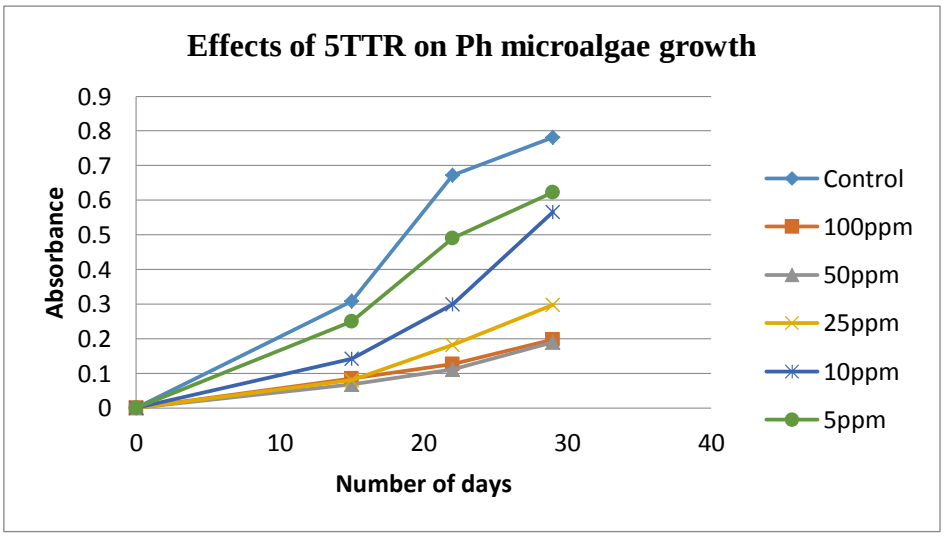


Figure 68 – Second test of microalga Ph growth rates with different concentrations of 5TTR in the medium

With the results on both tests we can conclude that the Ph microalga is sensitive to concentrations of 5TTR of 5ppm or higher and lower concentrations should be evaluated on further studies since that the results on the first test for lower concentrations than 5ppm were inconclusive. The EC_{50} estimated is between 25 and 10ppm.

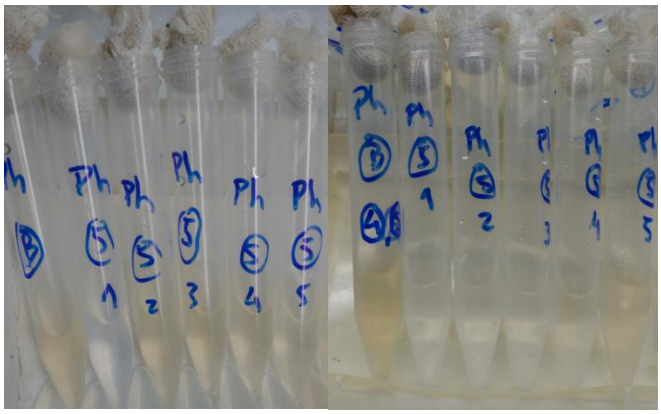


Figure 69 – Samples and control tubes of the first test (left) and second test (right) of the Ph microalga culture with 5TTR on the 19th and 22nd days, respectively

4 Discussion

Both macroscopic and spectrophotometric results allowed us to take some conclusions about the sensitivity of the D, K and Ph microalgae to the pollutants in study. The Ph microalga showed no sensitivity to the presence of the benzenesulfonamides in study (the parent compound BSA and its derivative pTSA) in the medium, for concentrations equal to or lower than 100ppm (the highest concentration evaluated in this study).

The effects of the BSA on the growth of the K microalga, the BT on both D and K microalgae and HOBT on Ph microalga were inconclusive, mostly because of the inconsistency between both tests performed, so further studies should be executed in order to understand what went wrong and what the real effects of the pollutants on the growth of these microalgae really are.

Although on the rest of the tests we achieved conclusive results, in some of them we didn't obtain results as precise as we expected. For example, since the second tests involving the K microalga were performed with the same range of concentrations as on the first one and on most of them the results for some concentrations weren't conclusive, the effects of these pollutants on the growth of the microalga should be evaluated on further studies with a different range of concentrations. Also, for the D microalga we suggest that the effects of concentrations equal to 50ppm or lower of HOBT should be evaluated in future studies to achieve more precise sensitivity value as well as for the Ph microalga concerning concentrations of 5ppm or lower of 5TTR.

With the results on this study we can conclude which microalgae are more sensitive to each pollutant, and so, the best choice as a biosensor for each pollutant. Since that the growth of the Ph microalga wasn't affected by the presence of BSA in the medium and the results for the K microalga were inconclusive, only the D microalga is suited to be used as a biosensor to perform water quality analyses in

which the BSA is suspected to be present in concentrations equal to or higher than 25ppm.

The results for the pTSA showed that the best choice as a biosensor would be the D microalga which is sensitive to concentrations of 1ppm or higher of pTSA in the water (while the growth of the Ph microalga was not affected by the presence of this pollutant in the medium and the K microalga showed a lower sensitivity, of 10ppm).

Concerning the benzothiazoles compounds the Ph microalga would be the best choice as a biosensor to evaluate the concentration of both BT and MeSBT in the water, in which the microalga was sensitive to concentrations of 10ppm or higher and 25ppm or higher respectively, and the K microalga for the HOBT for concentrations of 10ppm or higher. The results for the pollutant BT were inconclusive for the D and K microalgae and the Ph microalga demonstrated the highest sensitivity for the MeSBT (the K microalga was only sensitive to concentrations of 100ppm or higher and the D microalga to concentrations of 50ppm or higher, in comparison to the 25ppm of the Ph microalga). When it comes to the results of the effects of the HOBT on the growth of the microalgae the results were inconclusive for the Ph microalga and the D microalga was only sensitive to concentrations of 100pm or higher of HOBT (while the K microalga demonstrated a sensitivity of 10ppm).

All the microalgae had conclusive results for the benzotriazoles compounds. The Ph microalga was the most sensitive of all three microalgae to both BTR and 5TTR pollutants. For the parent compound BTR the Ph microalga was sensitive to concentrations of 25ppm or higher while the D and K microalgae demonstrated sensitivities of only 50ppm and 100ppm, respectively. For the 5TTR pollutant both D and K microalgae were sensitive only to concentrations of 100ppm or higher while the Ph microalga showed a sensitivity to much lower concentrations, of 5ppm or higher of 5TTR in the medium.

The Commission of the Europeans Communities adopted in 1996 a classification related to chemical toxicities. Compounds are considered as very toxic when the EC_{50}<1ppm, as toxic when 1ppm<EC_{50}<10ppm, as harmful when 10ppm< EC_{50}<100ppm and being non-toxic for aquatic organisms when EC_{50}>100ppm (45).

With the EC_{50} values estimated in this work we can classify the emerging pollutants studied in very toxic, toxic, harmful or non-toxic to the D, K and Ph

microalgae. The BSA is then considered harmful to the D microalga (EC_{50} between 25ppm and 10ppm) as well as the pTSA for the D and K microalgae (EC_{50} between 25ppm and 10ppm and between 100ppm and 10ppm respectively). The BT can also be classified as harmful for the Ph microalga (EC_{50} between 25ppm and 10ppm), the pollutant MeSBT as non-toxic for the D microalga (EC_{50} higher than 100ppm) and as harmful for the K and Ph microalgae (EC_{50} lower than 100ppm and between 100ppm and 50ppm respectively) and the HOBT also as harmful for both D and K microalgae (EC_{50} between 100ppm and 50ppm and approximately 10ppm, respectively).

BTR can also be considered as harmful for all three microalgae (EC_{50} between 100ppm and 50ppm for both D and Ph microalgae and lower than 100ppm for the K microalga) while the 5TTR pollutant can be classified as harmful for the K and Ph microalgae (EC_{50} lower than 100ppm for the K microalga and between 25 and 10ppm for the Ph) and non-toxic for the D microalga (EC_{50} higher than 100ppm).

We came across with some unpredictable problems that might have led to the inconclusive results of this study. The appearance of some initial sign of growth of the microalgae inside the tubes varied a lot from test to test (in some of them it happened on the 14^{th} day of culture while in others only on the 25^{th}, and these variations also occurred with the same microalga and same pollutant) which can be related to the fact that although the same volume of starter culture was added to the culture medium in all of the tests, this volume probably had different concentrations of microalgae cells, which wasn't controlled. Some of the tests took more than a month to finish, in order to obtain the three absorbance measurements which is not practical for some urgent water quality analyses. We also don't have explanation for the lack of concordance of some of the repeated tests, which led to inconclusive results.

In order to avoid these problems on future studies we suggest that the growth inhibition assays should follow well-known protocols and guidelines (9,36,39) that follow more specific criteria that can overcome the problem of the unpredictability of the initial growth and usually are carried out in only 72h total. We also suggest that the growth inhibition assays should be combined with another toxicity test like measuring the chlorophyll fluorescence emission or measuring the production of ROS, to better understand and try to find an explanation if the results are inconclusive.

5 Conclusions

The increase in worldwide water contamination with numerous emerging pollutants has become an emerging environmental concern due to their potential considerable ecotoxicities and associated health issues. Numerous emerging pollutants and their metabolites are found ubiquitously in all aquatic and their behaviour and ecotoxicological effects remain little known.

Seven different emerging pollutants, were tested throughout this study: BSA and its derivative pTSA, BT and its derivatives HOBT and MeSBT and BTR and its derivative 5TTR. These pollutants are high-volume production chemicals that are already considered to be ubiquitous water contaminants. These compounds were found in environmental waters at concentrations from a few ng/L to hundreds of µg/L. It is then extremely essential to develop measures not only concerning the disposal and elimination of all water pollutants but also to improve the methods of analysis.

Commonly these compounds are analysed by chemical methods but they have the main disadvantages of being time-consuming and expensive. Using biosensors is ideal for environmental monitoring because they are cost-effective, rapid, easy and can be used on-site for real time detection and analysis of contaminants in the field. In this work we used microalgae as biosensors due to their high sensitivity and for being good biological marker and an early warning system of the pollution in the ecosystem. Microalgae bioindicators also have short response times, reproducibility and require few pre-treatment of the samples which makes it possible to use them on the field to detect environmental contaminants.

The main goal of the present work was to assess the sensitivity of three different microalgae (D, K and Ph) to these seven emerging pollutants by performing algal growth inhibition effects in order to use them as future biosensors for these emerging

pollutants detection and monitoring in water environments. The growth inhibition assays were made using spectrophotometry and macroscopic observation.

The Ph microalga showed no sensitivity to the presence of the benzenesulfonamides in study in the medium. The effects of the BSA on the growth of the K microalga, the BT on both D and K microalgae and HOBT on Ph microalga were inconclusive so further studies should be executed. The effects of all the pollutants studied on the growth of the K microalga should also be evaluated on further studies with a different range of concentrations. Also, for the D microalga we suggest that the effects of concentrations equal to 50ppm or lower of HOBT should be evaluated in future studies to achieve more precise sensitivity value as well as for the Ph microalga concerning concentrations of 5ppm or lower of 5TTR.

The D microalga was considered the best choice as biosensor to perform water quality analyses in which the BSA is suspected to be present in concentrations equal to or higher than 25ppm. The results for the pTSA showed that the best choice as a biosensor would be the D microalga which is sensitive to concentrations of 1ppm or higher of pTSA in the water. The Ph microalga would be the best choice as a biosensor to evaluate the concentration of both BT and MeSBT in the water, in which the microalga was sensitive to concentrations of 10ppm or higher and 25ppm or higher respectively, and the K microalga for the HOBT for concentrations of 10ppm or higher. The Ph microalga was also the most sensitive of all three microalgae to both BTR and 5TTR pollutants with sensitivities of 25ppm and 5ppm, respectively.

According to the classification of the Commission of the Europeans Communities using the EC_{50} values estimated in this study, the BSA was considered harmful to the D microalga as well as the pTSA for the D and K microalgae. The BT was also classified as harmful for the Ph, the pollutant MeSBT as non-toxic for the D microalga and as harmful for the K and Ph and the HOBT also as harmful for both D and K microalgae. The BTR was considered harmful for all three microalgae as well as the 5TTR pollutant for the K and Ph microalgae and non-toxic for the D microalga.

In order to avoid some of the problems that we came across in this study, for future studies we recommend that the growth inhibition assays should be combined with another toxicity test and that they should follow well-known protocols and guidelines.

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